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GLYCEROL METABOLISM AND OTHER BIOCHEMICAL FEATURES ASSOCIATED WITH OVERWINTERING IN THE ADULT INSECT *Pterostichus brevicornis* (Carabidae)

A

DISSERTATION

Presented to the Faculty of the

University of Alaska in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

By

William D. Arvey, B.S., M.A.

Fairbanks, Alaska

May, 1974

GLYCEROL METABOLISM AND OTHER BIOCHEMICAL FEATURES

ASSOCIATED WITH OVERWINTERING IN THE ADULT INSECT

Pterostichus brevicornis (Carabidae)

RECOMMENDED:

Dr. Laurence Irving

Dr. Hans W. Behrisch

Dr. Ronald Smith

Dr. Darrell Williams

Dr. L. Keith Miller, Chairman
Advisory Committee

APPROVED:

Director, Institute of Arctic Biology

May 7, 1974
Date

Vice President for Research

10 May 74
Date

ABSTRACT

Overwintering adult P. brevicornis are known to tolerate freezing at low temperatures during winter hibernation, during which time free glycerol accumulates in the hemolymph. Seasonal levels of several metabolites as well as glycerol were followed to determine sources of glycerol as well as metabolic pathways used in its synthesis and degradation.

Maximum winter glycerol concentrations reached 13% hemolymph volume. Glycogen analyses indicated that the source of glycerol is carbohydrate. Fatty acid analysis showed a marked increase in desaturation during winter and with cold acclimation and it is suggested that the changes play a major role in winter cold hardiness. Hemolymph amino acid concentrations appeared not to vary seasonally to a great extent quantitatively or qualitatively. Proline was the predominent amino acid found at all seasons. Carbon from alanine, proline and glucose was shown by ^{14}C isotope feeding experiments to transfer into these three compartments during hibernation. Anaerobic metabolism during hibernation was not demonstrated. Fat content declined slowly by (8-10%) during hibernation and is not suspected to contribute significantly by glycerol synthesis. Activity of the enzyme α-glycerophosphatase was not found. Microclimate measurements of the preferred winter microhabitat showed that snow cover and other factors moderated temperature extremes to a great extent. It is suggested that cold hardening and winter survival in P. brevicornis entails the restructuring of many metabolic processes and is not only a function of glycerol accumulation. The details and mechanisms of these changes are not understood at the present time.

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INTRODUCTION

General Orientation

The aim of this dissertation is to provide further understanding of the processes that result in the winter survival of insects which inhabit cold northern regions. Our knowledge at the present time of the precise mechanisms involved in many aspects of winter survival seems not to be in the realm of real understanding of these processes, but rather, in many instances, to be at the level of simple description and conjecture. Insects of many groups, species, and life stages have been studied to determine how each has evolved its own particular strategy for survival of what might be called these unfavorable periods for reproduction and growth. Although many interesting and seemingly unique ways of dealing with cold survival have been illuminated by such studies, an integrated picture that carries with it some degree of universality with respect to the basic questions involved has so far not emerged. Basic questions still unresolved include the nature of freezing injury at the cellular level, the biochemical and biophysical basis of cold and freezing resistance in animals and the nature of the protective effects, if any, exerted by the so called cryoprotectants such as polyhydric alcohols, sugars, and various macromolecules. Mazur (1970) is the most recent author to review the present status of our knowledge and concludes that much work remains to be done on these interesting and complex problems.

Pterostichus brevicornis and Previous Studies

Pterostichus brevicornis is a small, (average weight 8 mg.),

flightless, carabid beetle belonging to the subgenus Cryobius. Ball (1963) lists 3 species groups comprising the subgenus, with a total of 51 species. Brevicornis is one of the groups, and consists of two species, including P. brevicornis. Both species of the brevicornis group are holarctic in distribution, and brevicornis brevicornis, the subspecies utilized in this work, inhabits an immense geographic area covering the entire polar basin except for Greenland, reaching a southern limit in eastern North America in the northern Great Lakes region. In Alaska and Canada the habitat type in which P. brevicornis is found includes both boreal forest floor and the tundra north of the tree line. Ball (1963) states,

> "The distribution of P. brevicornis across the north suggests survival of this species in at least three (glacial) refugia: eastern North America, south of the ice; or in a coastal refugium, in the Great Lakes area, and possibly somewhere north of the ice in the center of the continent; or in the Alaska-Yukon refugium."

It appears then that P. brevicornis has had a reasonably long history in northern environments and should be expected to possess cryobiological adaptations.

Other workers at the Institute of Arctic Biology have preceded this author in discovering and utilizing the value of Pterostichus for research in cold adaptation. Miller (1969), Baust (1970), Baust and Miller (1970, 1972) in a series of papers, established that P. brevicornis was indeed a species which exhibited many interesting adaptations. Among those noted were:

a) overwintering takes place in the adult stage

b) "cold hardy" individuals are extremely tolerant of cold, with survival noted after cooling to -80°C

c) freezing at moderate rates does not impair the survival of cold hardy individuals

d) glycerol accumulation and lowered supercooling and freezing points accompany the cold hardening process

e) glycerol levels are correlated with increasing tolerance to cold and freezing

Kaufmann (1969, 1971) examined the life cycle in P. brevicornis, concentrating on events which occur during the period of hibernation. (Hibernation is the term preferred to distinguish this form of winter dormancy in adults from true diapause which is normally associated with a period of developmental arrest at some stage of the life cycle.) Her findings showed that P. brevicornis in interior Alaska required from 14 to 36 months to complete the life cycle, depending upon the time of oviposition, asynchronous oviposition and subsequent asynchrony in development of young result in generation overlap, and overwintering can thus take place in eggs, larvae, pupae, or adult forms. This type of extended life cycle has been reported for other species of insects found in cold regions (Kaufman, 1969; Janetschek, 1948; Mani; 1962).

Cold Hardening and Freezing Tolerance

Until recent years freezing tolerance in insects was thought possible only for immature stages and many, such as Asahina (1966), thought that adult insects could not survive freezing primarily because of the suspected susceptibility of highly differentiated tissues to

frost injury. Insects inhabiting temperate regions apparently seldom overwinter as adult, reproductive forms, and usually pass this time in immature stages. At higher latitudes and altitudes, however, more than one warm season is often required to complete the life cycle (see Downes, 1965). This is perhaps the major reason that adults overwinter in these regions. It is becoming apparent that a great many species from many insect orders overwinter in adult stages in Alaska (Baust, 1970; Miller, 1973, unpub.). The abundance and diversity of insects and other poikilotherms in interior Alaska testifies to the fact that these animals are quite able to survive the temperature extremes that prevail here.

To avoid irreversible injury at subfeezing temperatures animals must either avoid actual freezing, or be able to tolerate internal ice formation. Avoidance can be accomplished by either moving seasonally into microhabitats where subfreezing temperatures are not encountered, or by supercooling. Supercooling may be defined here as liquids or tissues cooled below their normal freezing points, while they remain unfrozen, or in the liquid phase. Most biological solutions are capable of some degree of supercooling, and the phenomenon has been often observed in insects. Salt (1961) first remarked upon the remarkable ability of certain species to greatly extend the normal range of supercooling in winter, observing that weeks or months can be passed in such a state. While the supercooled state is thermodynamically only conditonally stable, Salt (1961) showed that supercooled insect tissues and fluids can remain so for long periods with the chance of spontaneous ice formation depending on the degree of

supercooling, the time duration, and the presence or absence of internal nucleating agents which can cause spontaneous freezing.

Freezing tolerance as shown by P. brevicornis, implies that while a certain proportion of the fluids become frozen, subsequent revival and viability are not impaired. Just how some animals are able to avoid injury from freezing is, as mentioned, not clearly understood. Cell and tissue injury and death result from general freezing in vertebrates and many invertebrates. However tolerance of extreme cold and very rapid cooling rates have been observed in Rotifera, Nematoda, Tardigrada and other less complex forms like bacteria and viruses (Crowe and Cooper, 1971). For these animals no previous cold hardening or preparation seems necessary to ensure survival, but the continuation of life after exposure to temperatures approaching absolute zero degrees Kelvin has not been reported outside of these primitive forms unless the process of cold hardening has taken place previously. Cold hardening occurs naturally in those animals that normally occupy cold regions, but whether it can be artificially induced in other animals as well is not known. Since it is well known that insects which are cold hardy in the winter months are suceptible to freezing injury at other times of the year, it is apparent that the process of cold hardening must take place before or perhaps during, the arrival of cold weather.

Glycerol as a Cryoprotectant in Insects

The independent and almost simultaneous discovery of glycerol accumulation, by Wyatt and Kalf (1957) in winter diapause pupae of

Hyalophora cecropia and by Chino (1957) in diapause eggs of Bombyx mori stimulated great interest especially in light of the findings of Polge, Smith, and Parkes (1949) that glycerol greatly reduced freezing damage in frozen spermatozoa. It was thought that perhaps insects, by internal metabolic processes were able to manufacture their own cryoprotective compounds and thus become resistant to the effects of freezing. Further studies on glycerol soon followed this discovery, (Salt, 1957; Wyatt and Kalf, 1958) and others. In all cases glycerol was found in the diapause stage of the insects studied, which was also the only stage known to overwinter in cold areas at that time. Thus it became a fairly strong belief that glycerol played a major role in cold hardening. The findings of Salt (1957, 1958, 1959) that glycerol levels correlated with the degree of supercooling, the freezing point depression of the hemolymph, and with survival after freezing strengthened these views. Salt (1957) reported that the larvae of one insect, Loxostege sticticalis was not freeze-tolerant even with significant amounts (2-4 % of wet weight) of glycerol, and he was thereby not convinced that glycerol alone could protect insects from frost injury. However in this case Salt (ibid.) does not report the conditions under which the insects were frozen, and it is possible that the freezing rate was too rapid or too slow for even cold hardy larvae to avoid injury. In this connection Mazur (1970) shows that the rate of freezing is critical for cell survival. Above optimum rates for any particular cell, can result in intracellular freezing while with below optimum rates deleterious osmotic, or solution effects can occur,

reducing cell survival. Salt (1959) showed that the depression in freezing point in the cold hardy larvae of Bracon cephi could not be entirely accounted for by the measured levels of glycerol, and suggests that other (unspecified) organic solutes must be responsible for part of the observed depression.

Possible Mechanisms of Glycerol Accumulation

Since the discovery of glycerol accumulation in insects, many attempts have been made to determine the metabolic mechanisms which control the phenomenon, because of the seeming importance of the compound as a cryoprotectant. In a brief attempt to pinpoint the source of glycerol, Salt (1959) found such little variation in levels of both glycogen and fat during periods of glycerol fluctuation in Bracon cephi that he considered neither to be probable precursors.

Chino (1957, 1958, 1960, 1961) in a series of papers made the first contributions of real value to this subject. In his studies of carbohydrate metabolism in diapause eggs of Bombyx mori, he postulated that glycogen was converted to glycerol and sorbitol at the onset of diapause, and when diapause was terminated glycogen was resynthesised. The mechanism proposed to carry out these transformations involved a blockage of terminal respiration in the cytochrome system at the onset of diapause. Glycerol and sorbitol formation result from the actions of three separate polyol dehydrogenase reactions in the silkworm egg. He found no evidence to indicate that sorbitol or glycerol might be formed from lipid precursors in Bombyx.

Faulkner (1956, 1958) reported a $NADP^+$-linked, polyol dehydrogenase

in the fat body, blood, and other tissues of the Bombyx silkworm. This enzyme was reported to reduce a number of hydroxyaldehydes and carbonyl compounds to their corresponding alcohols, including the direct conversion of glyceraldehyde to glycerol. Sømme and Velle (1968) made findings similar to those of Faulkner (1958) in Pieris brassicae and reported a single enzyme that reduces a number of substrates to their corresponding alcohols.

The reduction of dihydroxyacetone-phosphate (DHAP) to α-glycerophosphate is a well known shunt or alternate pathway in insects, whereby the DHAP acts as a hydrogen acceptor from reduced NAD, preventing the formation of lactate. This is an especially active pathway in insect flight muscles where the demand for energy is extremely high (see Weis-Fogh, 1967). α-glycerophosphate is a small molecule, and diffuses much more rapidly than the larger NADH through mitochondial membranes, insuring a quick supply of hydrogen for the aerobic machinery, thus avoiding the accumulation of large oxygen debts entailed when lactate is formed. Dephosphorylation of α-glycerophosphate gives rise to free glycerol and it was thought by Wilhelm et al. (1961) that under anaerobic conditions, glycerol would be formed as an end product. Working under the hypothesis of Chino (1958) that diapause entails at least partial anaerobiosis, Wilhelm et al. (1961) were able to show that anoxia in early diapause pupae of Hyalophora cecropia promoted an increase in glycerol levels and lactic acid, as well as some other unidentified metabolites. In addition they found that glycerol levels increased while glycogen stores decreased at the beginning of diapause in this species.

Sømme (1966) working with a flour moth, Anagasta, found that after 12 to 24 hours of anoxia, hemolymph levels of glycerol, lactate, glucose, inorganic phosphorus, and ninhydrin positive substances had increased, and that supercooling points of the hemolymph had decreased. In a separate study Sømme (1967) looked at the effects of anoxia in several species of diapausing insects and found a non-uniform response with regard to glycerol and other solute levels. In this connection, Kurland et al. (1958) subjected diapausing silkworm pupae of several species to anaerobiosis and found that they had great tolerance for anoxia, and that lactate accumulated and was reoxidized upon termination of anoxia. However the natural occurrence of anoxia during diapause was not reported. It is significant that oxidative processes were still present during diapause.

The relationship between anoxia and glycerol accumulation, and especially the question of whether anoxia normally occurs with diapause or hibernation is an area which deserves much more study. Notwithstanding the results of Wilhelm et al. (1961) and Sømme (1966), the work of Scholander et al. (1953) showed that Alaska midges (Chironomus) maintained measurable respiratory rates below 0°C., Burkett and Schneiderman (1968) found coordinated neuromuscular activity functioning to open and close spiracular openings in frozen moth larvae, and Salt (1958) demonstrated respiration in supercooled Chironomus larvae.

A preliminary examination of the effects of anoxia on glycerol accumulation in hibernating P. brevicornis has been carried out in this study, the results of which will be presented in a later section.

One of the few studies done on the phenomenon of glycerol accumulation in adult hibernating insects was that of Nordin et al. (1970), on the Carpenter Ant, Camponotus pennsylvannicus. This species has been shown to accumulate 2-4% glycerol/fresh weight, but cannot survive actual freezing. Nordin et al. (1970) attempted to pinpoint some of the factors important in the regulation, or metabolic control of glycerol synthesis. In addition to finding that glycogen levels decrease while glycerol levels increase, they also found that glyceraldehyde-3-phosphate dehydrogenase showed kinetic properties in Camponotus and in Apis mellifora (honeybee) such that enzyme activity continued at 0°C, a temperature which is known to inactivate yeast and mammalian enzymes.

The concept that metabolic pathways may be regulated in poikilotherms by enzyme systems that are differentially sensitive to the thermal environment is becoming well established. Hochachka (1967) suggested some possible ways in which an enzyme system could respond to thermal changes within the limits of the organism's temperature-tolerance range. One is that temperature directly affects the affinity of the enzyme for modulators, substrates, and products in such a manner that under a given set of temperature conditions, one pathway becomes operative, while under other temperature conditions the opposite pathway might be favored. This is especially likely at certain branch points in metabolism where, depending on which enzyme is favored, net metabolism either results in one direction, say degradation of storage materials and subsequent energy release, or in the opposite direction, say, synthesis

of storage products. Atkinson (1966) discussed these and similar possibilities with regard to the importance of allosteric effects, a concept first put forth by Jacob and Monod (1961, 1963). Central to this is the idea that conformational changes of the enzymes themselves, under the influence of positive or negative modulators could regulate the direction of metabolism in a cell or an organism. These ideas could be applied to studies of the metabolism of insects undergoing metabolic changes in response to temperature fluctuations, and may lead to the ultimate answer to the question of how polyol production is controlled in winter phase insects. Some preliminary steps were taken in this study to assess the effect of temperature on a key enzyme in the glycolytic pathway, fructose diphosphatase. If this enzyme is operative, the flow of carbon is from stored carbohydrate into the citric acid cycle with a subsequent release of energy. However if conditions and modulators favor the enzyme which mediates the flow of carbon in the reverse direction, i.e., phosphofructokinase, metabolism will proceed in the opposite direction with net synthesis of glycogen (see Behrisch and Hochachka (1969a, b). In theory, both enzymes should not be simultaneously active as this would result in a short circuit of metabolism, or a very inefficient metabolism yielding only a net hydrolysis of ATP (Hochachka, 1967).

Experimental Approach

Instead of concentrating on a single issue in this work, such as metabolic regulation of glycerol production, it seemed more profitable in view of our incomplete knowledge of the total process of cold

adaptation in arctic poikilotherms, to try to draw together a more complete picture of gross seasonal changes in many parameters, to see if obvious and important changes besides glycerol accumulation might be occurring. In addition to providing information about the precursors of glycerol, the detection of other factors important to the cold hardening process was attempted. There are many reasons why simple accumulation of a cryoprotectant such as glycerol should not be sufficient to afford complete protection from cold. For example, cold hardiness cannot be conferred upon a summer acclimatized insect by the injection of some given amount of glycerol (Takehara and Asahina, 1960). The working hypothesis used here is that winter survival must entail restructuring of many metabolic and physiological processes. With this in mind, a number of biochemical parameters were assayed and monitored seasonally.

It is known that free amino acids play a significant role in the energy metabolism of insects, and high levels are consistently found in hemolymph. The contribution made by these potential metabolites to glycerol metabolism and to cold hardiness has been little studied. Therefore, the attempt was made to both survey the amount and kinds of amino acids found in P. brevicornis throughout the year, and in addition, radioisotope techniques were used to determine whether certain amino acids are involved in glycerol synthesis. As organic solutes in the hemolymph, amino acids would contribute to cold hardiness simply on a colligative basis (although inefficiently) whereby freezing points and supercooling points would be lowered with increasing concentrations.

Thus it was important to determine whether major differences in total concentration could be observed at different times of the year while the insects were in different stages of thermal acclimatization. In addition the use of radioisotopes allowed us to assess the incorporation of amino acids into the glycerol pool and their potential importance as precursors to glycerol.

Changes in seasonal fatty acid composition have been observed or shown to occur for many species of vertebrates and invertebrates. The usual pattern observed is a trend toward decreasing fatty acid saturation with lowered temperature of cold acclimation or acclimatization. Buffington and Zar (1968), Pantyukov (1964), and Harwood and Takata (1965) all found decreased saturation of fatty acids in insects with lowered temperatures of acclimation. In addition Harwood and Takata (ibid.) noted that shortened photoperiod had a similar effect and Toombes (1966) suggests that the desaturation effect may not be due to cold stress per se but rather, is part of a general preparation for aestivation or hibernation. Studies of fatty acid changes are included in this study because of the often reported change in saturation with thermal acclimatization and because it is known that fatty acids are important constituents of cell membranes, which may be the critical point of injury and protection in cold adaptation (Mazur, 1970).

To determine which energy source might be responsible for glycerol synthesis in P. brevicornis, glycogen levels were followed seasonally with respect to hemolymph glycerol levels. Levels of glycogen and glycerol were also monitored during short term changes in acclimation.

Radioisotopes were used in this part of the study to see if the flow of carbon could be followed from glycerol to glycogen during periods of warm temperature acclimation. Following the studies of Chino (1957, 1958, 1960), Faulkner (1958), Sømme and Velle (1968), and Nordin et al. (1970), most workers are of the opinion that a modified glycolytic pathway based on the degradation of carbohydrate precursors gives rise to glycerol. It is possible, however, that other metabolites such as fatty and amino acids could enter into the reaction sequence. Therefore, labeled isotopes of alanine, proline, oleic acid, and glucose were fed to the insects in the attempt to check this possibility.

Finally, environmental temperature parameters were monitored in typical overwintering microhabitats of decaying stumps overlain by a winter's snow accumulation. Although P. brevicornis has been shown to possess great capacity for low temperature survival (Miller, 1969), the question has arisen as to whether such capability is necessary or desirable in the range of normal winter temperature regimes to which the overwintering individuals are exposed. It must be understood that in view of the extensive range and diverse habitat types occupied by P. brevicornis, stumps are certainly not the only possible hibernacula. No doubt it will be found that in other parts of the species range many interesting and diverse behavioral and ecological adaptations will be discovered which function to allow survival of long and often cold winters.

In retrospect it seems somewhat naive of me to have attempted an

advance on these issues by the study of an insect species. This is because insects, in the hierarchy of complexity exhibited in the animal world, represent, at least in the more advanced orders, extremely specialized forms of life with highly evolved biochemical, behavioral, ecological, morphological, and physiological systems. Insects no doubt are also endowed with the fundamental mechanisms that interest us in studies such as this, but the mechanisms often seem to be hidden, or overlain with complexities that many times are not a problem for the cryobiologist using red blood cells for example, as his experimental system. Nonetheless it is hoped that while the questions of fundamental importance may not be fully answered in studies of this type, perhaps a significant contribution will be made in a broader sense. More restricted questions concerning adaptation at many levels are therefore addressed.

METHODS AND MATERIALS

Collection and Habitat of P. brevicornis

Adult beetles were collected from decayed wood stumps commencing with the first hard freezes at night in late September and continued periodically until the snow melted in early or middle May. Adult P. brevicornis begin aggregating in winter hibernacula after the first frosts occur in mid-August and are all situated for the winter by about the first week in October. Small summer collections were also made by using pit fall type can traps, placed in depressions on the forest floor. Mr. H. D. Draper of Chatanika, Alaska, provided us with a constant supply of beetles from the surroundings of his log cabin on the Chatanika River at 40 Mile on the Steese Highway. This area contains many decaying stumps of black spruce (Picea mariana), white spruce (Picea glauca), and fewer, scattered stumps of paper birch (Betula papyrifera). The trees were cut some 60 years ago to support mining activities in this area, and are now in the terminal stages of decay. Many of the stumps are now overgrown with mosses, lichens, and other vegetation such as low bush cranberry (Vaccinium vitis-idea). The interior of the stumps varies as to the state of decomposition, but invariably has been tunneled at one time or another in the earlier stages by carpenter ants (Camponotus ssp.), which leave extensive channel networks throughout the stumps. Many types of animals utilize stumps for habitats in winter and summer including insects, nematodes, centipedes, and spiders. After being collected in winter, the beetles were placed in small cans, cardboard cartons,

or polyethylene bottles, packed with stump material, and left outdoors at ambient temperature until utilized in the laboratory. The cans and bottles were placed in a large wooden box, which, when covered with snow resembled the normal winter thermal conditions of the insects.

Glycerol Determinations

To provide baseline information on the status of glycerol accumulation in Pterostichus and as a marker for stage of cold-hardiness, glycerol hemolymph levels were measured periodically during the fall, winter, and spring of 1969-1970, 1970-1971, and 1971-1972. Ascending paper chromatography as described by Perkins and Aronoff (1959), slightly modified by Salt (1959), Sømme (1964) and Baust (1970) was used. Strips of Whatman #1 filter paper (20 cm x 40 cm) were cut to width and then spotted with the sample to be analyzed, along with standards for comparison, and hung in an enclosed tank containing the solvent: n-butanol-acetic acid-water (12:3:5 v/v). Paper spotting was done with a one microliter Hamilton syringe. After 15 to 20 hours in the solvent the paper was removed, dried under a laboratory hood, then lightly and evenly sprayed with an atomizer containing .01 M potassium periodate solution. The paper was again dried, and then oversprayed with a solution containing 35% saturated sodium tetraborate, 0.8% potassium iodide, 0.9% boric acid, and 3% soluble starch. Polyhydric alcohols then appeared as white spots on a blue background. Comparisons of spot size were done by first cutting out the spot, then weighing it on a precision Mettler balance, accurate to one hundreth

of a milligram. A curve was constructed using the standards against which the sample glycerol amount was compared. Quantities of glycerol can be detected as low as 0.1 of one per cent glycerol in one microliter of solution, which is approximately equal to 0.1 M solution of glycerol which corresponds roughly to 9.2 micrograms of glycerol. The plot of glycerol concentration versus spot weight was curvilinear, representing a log function. Hemolymph samples to be analyzed were prepared by two different methods for paper chromatography. The method used by Baust (1970) and others involves simply a withdrawal of one microliter of hemolymph from the beetles, using a 1 microliter syringe mounted on a micromanipulator stage. This operation was performed under a binocular dissecting scope with illumination. The insect was placed ventrally on a restraining bed of clay, while the cuticle of the thorax was punctured with a needle or pin. Pressure was exerted on the dorsal area which caused hemolymph to exude from the thorax puncture. The hemolymph was then sucked into the syringe. The amount of hemolymph obtained from an individual beetle generally fell in the 0.3 to 0.4 microliter range depending mainly on the state of hydration. Thus 3 or more individuals were often needed to obtain one full microliter. Results were then expressed as glycerol per cent of one microliter hemolymph solution. Conversion is also possible to micrograms per gram, or some other concentration function such as molarity. The second chromatograph procedure involves first weighing a number of beetles (30 was found to be convenient), and then homogenizing them in 70 percent ethanol using

a Virtis high speed homogenizer. The homogenate was then centrifuged at 10,000 G for 15 minutes. The supernatant poured off into a separate tube, 2 more ml of ethanol were added, the tube recentrifuged and the supernatant saved. The combined supernatants were dried in a desiccator or over an air stream. Water is added to equal the amount of the original body water and a 1 microliter sample was spotted onto the paper strip. The spot size which results from the developed chromatograph could be related directly to body weight. When this method was utilized, glycerol was reported as a per cent of body water, not total weight. To test whether the results of glycerol analysis using paper chromatography were reliable, a gas-liquid chromatographic procedure was employed periodically. The procedure, slightly modified, was taken from Nordin et al. (1971). One microliter hemolymph samples were taken by cuticle puncture and placed into conical 15 ml test tubes containing 0.20 ml pyridine and 0.20 ml acetic anhydride. Standards were prepared at the same time. All tubes were incubated at 70°C for 15 minutes to acetylate the glycerol completely, then the excess solvent was dried off in a stream of nitrogen or air. No prior sample preparation such as deionization with resin columns was necessary because the presence of salts, proteins and other hemolymph constituents did not affect the final results. The residue after drying was dissolved in 0.20 ml of chloroform, and then analyzed by gas chromatography for the glycerol triacetate content. We used an F and M model research chromatograph equipped with twin flame ionization detectors, and a 6 ft column of stainless steel measuring 0.125 inches in diameter. The column

contained 3% ECNSS-M, Applied Science Laboratories Inc.), on a chromosorb Q support. Column temperature was kept at 135°C while one microliter of sample was injected. Glycerol triacetate peak area was measured using a Honeywell chart recorder. Results are compared to standards which were run every time and tabulated as percent glycerol in 1 microliter hemolymph samples.

Fat Determinations

Total lipid was measured, as well as the fatty acid composition of the whole insect. Total lipid analysis was done using the method of Karnavar and Nair (1969). Samples of about 100 insects without regard to sex or age class were weighed, then homogenized in ethanol-ether (3:1 v/v). The extract was centrifuged, and the residue rewashed first with the original solvent, recentrifuged, then washed with petroleum ether and centrifuged for a fine time. The combined supernatant was evaporated to dryness at about 60°C, taken up in petroleum ether and redried. After this the fat was weighed.

Fatty acids were prepared according to a modified procedure of Metcame and Schnits (1961). About 100 insects were freeze dried for 24 hours. Fat was extracted from the dried, ground up beetles in petroleum ether using a Soxlet extractor for 8 hours. The extract was dried, saponified, and then converted to free fatty acids by adding HCl. Methylation takes place by adding boron trifluoride to the tubes while kept at 100°C in boiling water. The sample was passed over sodium sulfate to remove water and evaporated to near dryness. One tenth to 0.2 microliters were injected into the chromatograph.

The column used was 20% DEGS, 8 ft long, 0.125 inches diameter. Temperature was kept at 197°C with a gas flow rate of 1.3 ml/min. Standards were used to identify some of the fatty acids. Identification was done using semilog paper and standards to generate series of parallel straight lines according to the degree of saturation. Fatty acids with different numbers of saturated bonds fall on separate but parallel lines (Ackman, 1963). Some minor fatty acids, and isomeric forms were not identified with precision since the object of the study was to detect gross changes in composition which would occur seasonally or with different acclimation temperatures.

Glycogen

Glycogen content was periodically measured by the procedure of Nordin et al. (1971). The insects (25-30) were weighed, homogenized, and extracted in 10% ice cold trichloracetic acid 2ml/100mg fresh weight). The suspension was centrifuged at 10,000 G for 15 minutes, and the supernatant then transferred to a 15 ml test tube in ice. The pellet was washed once with 2 ml TCA, recentrifuged and the supernatants combined. To this solution was added 1.2 volumes of cold absolute ethanol and the solution was then allowed to sit in the refrigerator 12-18 hours. The sample was centrifuged, the supernatant discarded, and the pellet was redissolved in water. After reprecipitating and dissolving the glycogen pellet twice, the sample was dissolved in 5 ml of water. Glycogen content was then measured by the phenol-sulphuric acid method of Dubois et al. (1956). Two

ml aliquots were placed in test tubes and 50 microliters of 80% phenol solution (80 gm phenol, 20 gm water) were added. Five ml of sulphuric acid was rapidly pipetted into the tube. The concentration of glycogen was measured spectrophotometrically at 490 nm, and compared to a standard curve. Results were expressed as mg/gm, fresh weight.

Amino acids

Amino acid analyses were performed with an automatic amino acid analyzer (Technicon, Chauncey, New York) built from the design of Spackman et al. (1958). The principal modifications were the use of the single column gradient system of Piez and Morris (1960) and the Technicon roller type pump. Free amino acids from one microliter of hemolymph could thus be quantitatively analyzed, without prior sample preparation. All analyses were done by Ms. Andrée Porchet, Institute of Arctic Biology Technician.

Isotopes

Isotope techniques were utilized to attempt to follow carbon (^{14}C) flow through different transformations of substrates. The small size (8 mg average weight) of the beetles made quantitative injection of labeled precursors extremely difficult, so ingestion during periods of active feeding was used. Confining the beetles in the presence of food containing labeled substances made it possible to introduce small amounts of ^{14}C labeled materials into the digestive tract.

In the first series of trials, insects previously frozen at ambient temperatures outdoors in winter were allowed to thaw and begin

acclimating to the temperatures in the laboratory (20-22°C). After sufficient time had elapsed, ^{14}C labeled glycerol was administered in aqueous solution, absorbed into sections of paper towel. The specific activity of the glycerol was 2.7 microcuries/25 ml. Over a period of several days thereafter, glycogen was extracted from the beetles and counted for its ^{14}C content by liquid scintillation. The scintillation mixture consisted of 5 gm/liter PPO (2,5 diphenyl-oxazole) and 0.1 gm/liter dimethyl POPOP (1,4-bis-2-4 methyl-5 phenyloxazolyl-benzene in toluene). When counting aqueous solutions of glycogen, Triton-X 100 was added (3:1) as a wetting agent. Samples were counted with a Nuclear Chicago Mark I liquid scintillation system. Each sample was counted for a minimum of 3 count periods, each 10 minutes in duration. Variability of counting efficiency due to quenching was evaluated by external standardization, and sample counts were corrected for background rates which ranged from 15-25 disintegrations per minute (dpm). The counting efficiency ranged from 75-80%, i.e., 75-80% of the ^{14}C decays were detected.

The next series of isotope experiments involved feeding labeled substrates of D-glucose, with uniform ^{14}C label, oleic acid with uniform ^{14}C, L-alanine and L-proline both with uniform label. The compounds were purchased from Amersham/Searle Corp., Arlington Heights, Illinois. Groups of about 100 individuals were contained separately and given a small slice of apple into which the labeled compounds were injected more or less uniformly. The dosages were as follows: oleic acid, 30 microcuries; proline, 50 microcuries; alanine 25 microcuries;

glucose, 25 microcuries. The introduction of labeled food took place in the first week in October, after the first seasonal frosts, but before the beetles had completely ceased feeding. The containers were then kept in a large wooden box, outdoors, exposed all winter to ambient temperatures. Unknown but sufficient amounts of labeled substrates for counting purposes were thus ingested by individual beetles prior to the winter period of complete inactivity. Direct injection of known amounts of ^{14}C labeled materials was previously found to be unfeasible due to the small size of the insects, and to the small number of individuals which we were able to treat with reliable results in this manner.

To determine incorporation of substrates into glycerol, the following procedure was followed: first, hemolymph samples were obtained in 1-3 microliter quantities, and spotted on No. 1 Whatman filter paper strips. The strips were then run in solvent (n-butanol: acetic acid:water, 12:3:5), dried, and sprayed to visualize glycerol spots. The chromatogram was cut into squares measuring 2 x 2 cm along the path of migration of the spot, starting 1 cm below the origin of migration, and ending 2-4 cm above the glycerol spot. The squares were given numbers according to their position of the strip, and then counted with liquid scintillation. Relative activity could thus be assessed for any position on the chromatograph strip. This procedure was also followed in reverse when looking at beetles in the process of de-acclimation. Outdoor acclimatized winter insects were taken into the laboratory at 22°C and allowed to acclimate over

a period of time. During this time hemolymph was chromatographed to determine whether decreasing glycerol amounts could be correlated with increasing label in other hemolymph constituents.

Enzyme Studies

Preliminary studies were undertaken on enzymes thought to be involved in glycerol formation. The enzymes studied were α-glycerophosphatase, (α-GPase), and fructose diphosphatase, (FDPase). α-GPase is responsible, or potentially responsible for hydrolyzing inorganic phosphate from glycerophosphate in what may be the terminal step in the pathway to glycerol. FDPase, another glycolytic enzyme, operates at a key point in glycolysis by reducing fructose-1-6-diphosphate to fructose-6-phosphate, and is thus involved in the sequence which results in the formation of glucose or glycogen.

Enzymes were studied in both winter and summer insects, and in cold acclimated and warm acclimated beetles. The general procedure for assaying enzyme activity was as follows: from 30 to 100 live beetles were selected depending on the volume of enzyme preparation required, weighed, and then homogenized in a Virtis homogenizer in water at 0°C for 2-4 minutes. Mixtures of 10% (wt/volume) were used. The homogenate was then centrifuged at about 10,000 G in a refrigerated centrifuge at 0-5°C. The supernatant was saved as the enzyme preparation. It was found that freezing did not destroy enzyme activity and samples could be kept frozen for several days. Activity from sample to sample could be compared either from tissue weight per volume comparisons or from total amounts of protein in the preparations. Protein determinations were made

according to the method of Murphy and Kies (1960).

Reaction mixtures of 2 ml final volume were incubated in 5 ml disposable test tubes placed in a temperature controlled water bath. Tris (hydroxymethylaminomethane) Hcl buffer was used to control pH, and constituted most of the mixture volume. Magnesium chloride or Manganese chloride were used as ionic cofactors in the reaction. Standard concentrations of substrate, either FDP or α-GP were prepared and added to the mixture. When cofactor buffer and substrate had all been added in the proper proportions, the tubes were allowed to equilibrate in the water bath to the temperature desired. Then the enzyme preparation was pipetted quickly into the tubes, after which, 0.5 ml of 10% trichloroacetic acid (TCA) was added to 0.5 ml of the reaction mixture to stop the reaction. Values of inorganic phosphate obtained at this time were taken as baseline or zero time values. The reaction was allowed to proceed for a half hour or one hour and again 0.5 ml was removed and placed in the TCA. The activity of the enzyme was reflected in the different amounts of free inorganic phosphate obtained between time zero and the end time for the reactions.

To assay the liberated inorganic phosphate, a Technicon autoanalyzer consisting of sampler, proportioning pump, colorimeter and chart recorder, was used. The autoanalyzer uses a modification of the Fiske and Subbarow procedure (1925) for determination of inorganic phosphate (Pi). It is based on the formation of phosphomolybdic acid which is reduced by 1-amino-2-napthol-4-sulfonic acid. After heating in a temperature bath, the mixture produces a blue color whose optical density was proportional

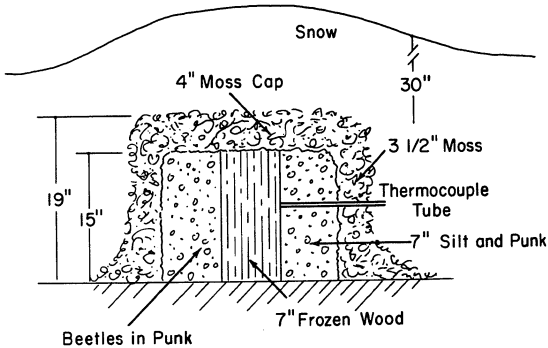
to the amount of phosphate present. This colored product was read by the machine at 660 nm in a tubular flowcell with a 15 mm light path. Results are then expressed as moles of inorganic phosphate released per hour per ml of enzyme preparation.

Microclimate

Information was needed on the microhabitat occupied by overwintering beetles. From the collection area on the Steese Highway, with the help of Mr. Draper, we have assembled seasonal temperature data including daily maximum and minimum temperatures. In addition, we embedded thermocouples in 2 typical, natural stumps inhabited normally by P. brevicornis during the winter (Fig. 1). This was done in one stump by taping thermocouple leads at measured depth intervals onto a long thin bamboo stick, which was then inserted into the stump to a known depth in the fall before the stump had frozen. In another stump, six thermocouple leads were inserted into a hollow glass rod, whose distal end had been sealed. The thermocouples were placed at measured depths as with the other probe. With the probes imbedded in the stumps, temperatures at different stump depths were taken almost daily with a portable potentiometer. The effect of snow cover and wood depth on microhabitat temperatures could thus be assessed.

The first stump wired originally had 5 thermocouple leads implanted. Insertion depths of the leads on the probe bar were 35cm, 26cm, 14cm, 9cm, and 2cm. The leads placed inside the tube on the second probe were placed at corresponding depths, with an additional lead at 0cm depth.

Fig. 1 Schematic drawing of winter habitat for P. brevicornis. Shows stump, surrounding moss and punk with snow cap. Thermocouple probe permanently implanted to approximate depth shown. All measurements shown in inches.



A sketch of the stumps, moss layer, snowcap, and their dimensions is presented in Figure 1. Thermocouple probes were inserted through the moss layer, and through the outer stump layer which consisted of punky, channeled wood to the harder interior wood through which the probes would not penetrate. In both cases the outer thermocouple leads were situated very near the moss/air interface, where they were subsequently covered with more than 30cm of snow.

RESULTS

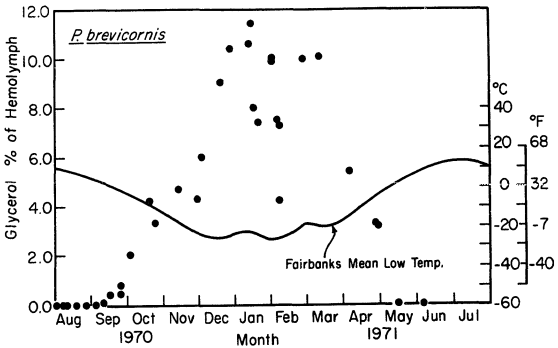
Glycerol

Figure 2 summarizes the yearly fluctuation found in hemolymph glycerol concentration in P. brevicornis. From this it can be seen that there is a definite time correlation involved with glycerol accumulation. Levels begin to increase near the time of the first frosts in September, at which time P. brevicornis adults begin to locate themselves in their winter hibernacula, decaying stumps. It appears that all adults are located for the winter before the "hard frosts" begin, i.e. before nighttime temperatures consistently reach into the -5 to -10°C range. Our observations indicate that once the insects are so sequestered in these microhabitats, they remain until snow in the spring is completely gone from the surroundings, and temperatures are consistently above freezing. This represents a period of inactivity of somewhat over 7 months.

After the second week in September, glycerol levels begin to rise in the beetles and by the first week in October, a level of 3 or more percent is attained in the hemolymph (3% of hemolymph volume). Accumulation continues until sometime in January, after which time no further increases are noted in percentage composition. Levels fluctuate very little until near the middle of April. Then glycerol begins to decline and by the first week in May, levels approach zero percent.

Included in Figure 2 for comparison are the annual mean low temperatures at Fairbanks, Alaska, compiled and published by the National Weather Service.

Fig. 2 Seasonal glycerol levels of P. brevicornis (solid dots) from August to July 1970-71. Glycerol expressed as percent of hemolymph by volume. Solid line represents published mean low temperature at Fairbanks, Alaska.



In this study, and in the work of Baust (1970), and Baust and Miller (1972), it was noted that after increased glycerol levels were attained in the fall and winter, reacclimation of the insects to warm temperatures was accompanied by a steady and rapid decline in hemolymph glycerol concentration. Insects taken from outside on January 11 were checked for hemolymph glycerol concentrations over the following 72 hours with the resulting decline in glycerol shown in Figure 3. The slope of the regression line predicts that at 5°C, glycerol levels reach zero after approximately 88 hours and depend upon the temperature as Baust (1970) has shown.

Cold hardiness, defined here as the ability to withstand freezing without permanent impairment, is lost at a similar rate when the insects are kept at the same temperature (5°C). Figure 4 shows the percent survival of insects reexposed to environmental temperatures during the winter after spending various lengths of time at 5°C.

Glycogen

Figure 5 records glycogen levels in micrograms per milligram of fresh weight over a one year period. An apparent trend is observed, with very low amounts recorded during the winter and summer months, and with greater amounts found in spring and fall. It can be noted by comparison with Figure 1 that at times when glycerol levels are elevated, glycogen levels are depressed, in a reciprocal fashion. The highest values observed for glycogen in naturally acclimatized beetles was 12 mg/gm in early September.

When cold hardened beetles are placed in an environment of 5°C and

Fig. 3 Rate of glycerol disappearance from hemolymph of winter acclimatized P. brevicornis when warmed to 5°C. Glycerol expressed as micromoles per microliter of hemolymph.

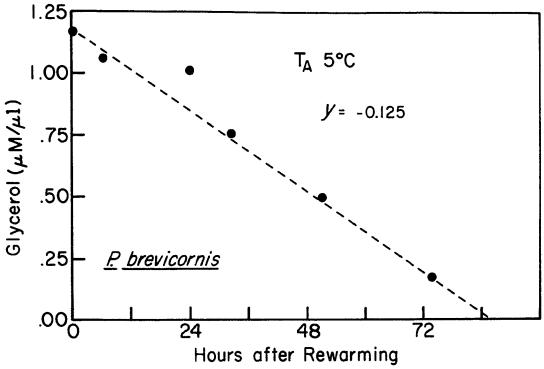


Fig. 4 Survival of winter acclimatized P. brevicornis after warming to 5°C for various lengths of time, followed by refreezing to outdoor ambient temperatures.

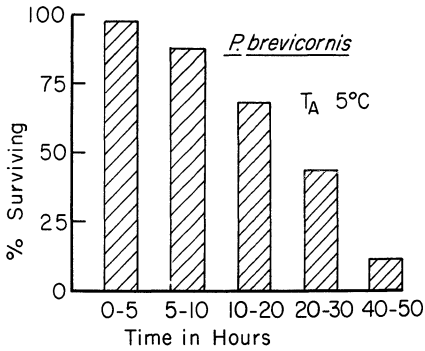
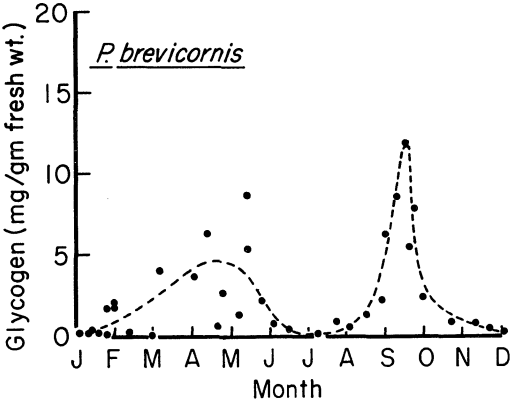


Fig. 5 Seasonal glycogen levels in P. brevicornis, expressed as milligram per gram of fresh weight. Curve is fitted by eye.



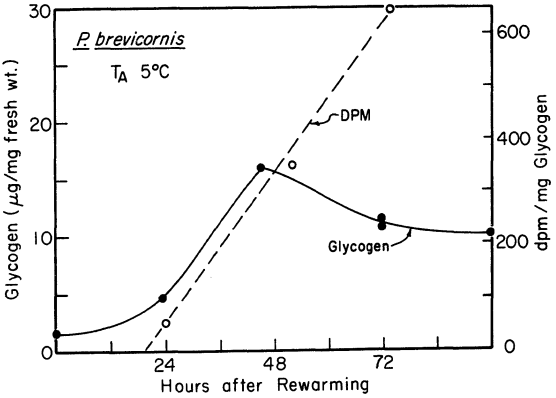
allowed to acclimate for several days, glycogen levels increase dramatically as shown in Figure 6. From amounts which are barely detectable, levels over 16 mg/gm of fresh weight are recorded after 48 hours. This increase coincides with a similar rapid decrease in glycerol. Figure 6 also shows a decline in glycogen levels starting about 48 hours after rewarming to 5°C.

To determine whether the increasing glycogen concentrations could be coming from glycerol carbon, beetles were kept during the acclimation period with paper impregnated with water and ^{14}C labeled glycerol. After thawing and regaining normal activity the beetles congregated on the moist paper and drank the aqueous glycerol ^{14}C solution directly from the paper. Glycogen was extracted afterwards at time intervals and analyzed on the liquid scintillation counter for the presence of ^{14}C. A progressive increase in the total activity of the glycogen sample was observed over a time period of up to 72 hours as shown also in Figure 6. This was an indication that glycogen can be synthesized with the incorporation of glycerol carbon in P. brevicornis. No subsequent decline in dpm/mg is observed, in contrast to the data given for whole body glycogen analysis.

Amino Acids

A total of 16 free amino acids were identified in the hemolymph of P. brevicornis and are tabulated in Table 1. Proline was found to be the predominant acid at all times of the year, comprising from 39.5 to 67% of the samples examined in terms of absolute concentration in micromoles. Alanine, histidine, lysine and arginine were the only

Fig. 6 Glycogen content of P. brevicornis after rewarming to 5°C (solid dots). dpm/mg of glycogen counted in glycogen extract of P. brevicornis after rewarming to 5°C (dashed line).



others found to be in amounts significantly greater than trace levels. Some, such as methionine and tyrosine were only found in one or two samples in very small concentrations.

Seasonal changes in percentages of different acids are not readily apparent. Histidine and arginine concentrations appear to increase significantly during the summer months, whereas alanine tends to be lower in the summer and more abundant in the winter.

In Table I the micromolar amounts in each sample have been tabulated, in an attempt to determine whether the absolute titer of amino acids in the hemolymph shows discernable changes. The mean for the 21 total samples is .424 micromoles per microliter with a standard deviation of .0256 micromoles and a coefficient of variation of 0.6039. Thus the total assemblage of samples exhibits extreme variation. Unfortunately the number of samples in each monthly period is too small for statistical analysis. There is perhaps a tendency for increased micromolar concentrations in the winter months which could only be verified with many more sample analyses throughout the year.

Fat, Water and Fatty Acids

Whole body fat and water determinations are illustrated in Figure 7 where results are related to dry body weight. Major seasonal trends cannot be conclusively shown from the data presented here because too many data points are missing in the summer and spring. Fall and winter fat levels however, seem to fall mostly between 15 and 20 percent of dry body weight. The few summer samples that were analyzed indicate a possible decrease in fat content during this time.

TABLE I

Free hemolymph amino acids
(percentage by month)

Acid	Jan N=2	Jun N=3	Jul N=2	Aug N=5	Sep N=4	Oct N=2	Dec N=3
threonine	tr	3.4	2.1	2.0	2.6	1.3	1.5
serine	1.5	4.2	-	-	3.9	3.0	2.2
glutamine	2.3	5.4	-	-	3.6	1.0	0.7
proline	61.7	48.6	39.5	53.2	50.0	67.0	57.5
glycine	2.0	4.1	4.7	7.9	4.9	2.8	2.5
alanine	13.1	9.1	5.5	7.0	12.4	9.2	10.2
valine	3.4	4.8	6.9	4.3	3.7	2.1	5.3
isoleucine	1.5	3.0	-	1.7	1.3	0.9	2.6
leucine	1.4	5.0	-	3.0	1.7	0.6	1.0
phenylalanine	1.2	-	-	3.4	2.5	0.46	0.3
lysine	3.5	6.4	3.6	5.3	5.2	4.4	6.9
histidine	3.0	6.3	19.7	13.1	4.3	3.2	5.2
arginine	4.0	7.3	22.0	7.7	2.5	3.1	4.1
asparagine	tr	2.2	2.0	tr	0.9	0.23	tr
methionine	-	1.7	-	-	-	-	-
tyrosine	-	2.9	-	-	-	-	-

Total amino acid
(micromole/microliter of sample)

Jan	Jun	Jul	Aug	Sep	Oct	Dec
.814	.370	.207	.235	.467	1.201	.238
.513	.254	.290	.190	.373	.278	.646
	.571		.283	.388		.689
			.193	.556		
			.159			

Fig. 7 Seasonal fat and water content of P. brevicornis. Fat is expressed as percent of dry weight.

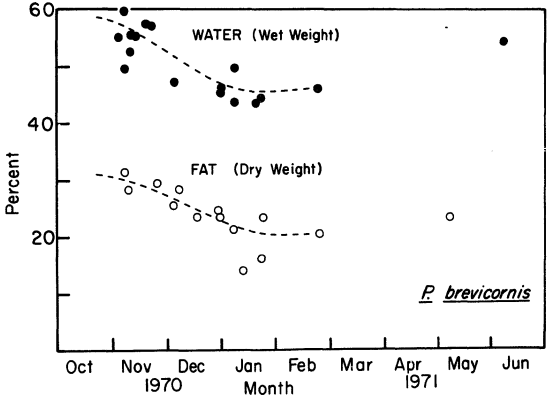


Table II lists the fatty acids identified or tentatively identified in the course of this study. An asterisk is placed beside those found consistently in major concentration. Those so marked are considered in Table III where percentages are calculated of individual acids in a given sample. As is shown, the acids 18:I: oleic, and 18:2: linoleic, comprise the major proportion of the total fatty acids, followed by palmitic: 16:0, and palmitoleic: 16:1. Rather marked changes are noted seasonally in the percentages of 18:1 and 18:2 with a definite trend towards increasing unsaturation during the winter months.

Short term acclimation to warm temperatures during the winter likewise brings about a definite decrease in the 18:2/18:1 ratio as is shown in the columns marked **. In the warm acclimated samples it is interesting to note that the 16:0/16:1 ratio did not change as much as the 18:2/18:1 ratio. Normally there does appear to be a seasonal shift in the palmitic/palmitoleic ratio, tending to be lower, i.e., more unsaturation, in the winter.

The ratio of saturates to unsaturates (S/U ratio) is included at the bottom of Table III. This ratio is calculated as a percentage of the total saturated versus the unsaturated, fatty acids from each sample. This gives a rough indication of the extent of seasonal saturation change. This value ranges from 0.04 in winter to 0.70 in the summer. The greatest amount of seasonal change appears to involve linoleic acid. Values in its percentage of the total sample range from less than 10% in the summer to over 50% in the winter. Changes in the proportion of this one acid alone may account for the major part of the total

saturation change noted.

Radioisotope Feeding

Of the four isotope substances fed to the beetles in early fall, all but oleic acid were ingested. The reason for the failure of oleic acid to become incorporated is not fully known, however it appeared that the foodstuff which contained this isotope was not palatable to the insects for some reason. ^{14}C counts above background were never detected from the hemolymph of those beetles which had been fed labeled oleic acid.

Proline, alanine and glucose on the other hand were ingested in small amounts by some of the beetles because hemolymph samples taken from October to March usually contained detectable amounts of labeled carbon.

Pooled one microliter hemolymph samples were taken from each isotope fed group at intervals throughout the winter from October to March. Four sampling periods are represented in Figure 9. As previously noted, liquid scintillation counting was done from chromatogram strips. On the ordinate of Figure 9 is percentage of counts above background levels, of a particular area of the chromatogram strip.

The procedure used for estimating ^{14}C incorporation was based on the fact that chromatographed hemolymph samples of ^{14}C fed insects tended to include 3 distinct areas or peaks of activity along the chromatogram strip. One was very near the origin, another near the 9cm position and the last was located near the 16 cm position, using the origin as baseline. The normal spot test for glycerol showed the last peak to be that compound.

TABLE II

Fatty acids found in P. brevicornis
(whole body extracts)

Fatty Acid Name	Carbon Number	Double Bonds
undecanoic	11	0
dodecanoic (Lauric)*	12	0
dodecenoic	12	1
dodecadienoic	12	2
tridecanoic (Trideane)	13	0
tetradeanoic (Myristic)*	14	0
tetradecenoic*	14	1
tetradecadienoic	14	2
pentadecanoic	15	0
hexadecanoic (Palmitic)*	16	0
hexadecenoic (Palmitoleic)*	16	1
heptadecanoic (Margaric)	17	0
octadecanoic (Stearic)*	18	0
octadecenoic (Oleic)*	18	1
octadecadienoic (Linoleic)*	18	2
octadecatrienoic (Linolenic)*	18	3
nonadecanoic	19	0
eicosenoic (Arachidic)	20	0
eicosadienoic	20	2
eicosatrienoic*	20	3
eicosatetraenoic (Arachidonic)*	20	4
heneicosanoic	21	0
docosanoic (Behenic)	22	0
docosenoic (Erusic)	22	1

*denotes fatty acids present in significant quantities

TABLE III

Fatty acid composition of P. brevicornis

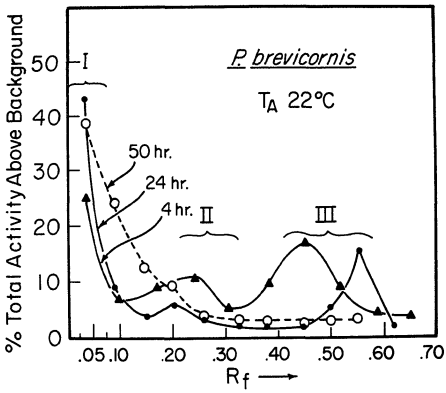
(Samples listed by date versus percent composition of each acid in sample)

Fatty Acid	Date (month/day) 5/3	6/22	7/6	7/9	12/4	12/18	12/9	1/14	12/14**	12/16**
12:0	0.19	2.4	5.2	0.5	0.15	0.46	0.24	tr	0.23	1.47
14:0	1.84	0.8	4.7	5.0	1.74	2.6	1.8	1.97	3.3	2.46
14:1	1.39	tr	0.63	0.6	1.46	1.4	0.86	0.95	2.5	1.82
16:0	7.05	17.0	24.2	25.0	1.84	1.65	3.3	2.83	6.3	4.3
16:1	1.68	5.2	6.27	4.2	10.6	15.5	12.3	12.4	17.0	14.9
18:0	0.74	2.4	7.16	7.8	0.63	0.93	1.06	0.42	1.1	1.65
18:1	24.9	60.4	42.4	47.8	26.5	35.8	29.2	29.5	36.5	37.0
18:2	43.7	11.3	7.5	8.2	56.0	49.0	50.0	49.5	28.8	31.0
18:3	1.9	tr	0.52	0.8	1.7	4.5	1.4	2.4	2.0	3.0
20:3	0.6	tr	0.7	tr	1.4	2.9	tr	tr	1.2	1.8
20:4	0.7	tr	0.7	tr	1.5	2.7	tr	tr	1.5	2.1
S/U*	0.10	0.29	0.70	0.62	0.04	0.05	0.06	0.05	0.12	0.10

* ratio of saturated to unsaturated fatty acids by percentage

** insects allowed to rewarm at 5° for several days

Fig. 8 ^{14}C distribution in chromatogram of hemolymph from P. brevicornis. ^{14}C originally fed as alanine with uniform label. Ordinate: percent of total sample ^{14}C dpm above background. Abscissa: R_f (distance from origin of ^{14}C relative to solvent front). Samples of hemolymph chromatographed after 4, 24, and 50 hours of rewarming at 22°C. I, II, and III: major activity areas of chromatogram.



The intermediate peak of activity had R_f values (ratio of migration distance of spot relative to migration distance of solvent) very close to those of the amino acids proline and alanine. Published values (Zweig, 1972) indicate R_f values of 0.30 for alanine, and 0.34 for proline in the n-butanol:acetic acid:water 12:3:3 v/v solvent system. In practice, however, I found the R_f for both acids to be very close to 0.26. This was calculated using the ^{14}C forms of the amino acids to allow close comparison with the trials using hemolymph. The actual R_f tends to be variable, within certain limits, and depends upon the length of time that the paper resides in the solvent, temperature and humidity of the tank, type of paper, and perhaps other factors. The attempt was made to be consistent in the control of all these factors but some variation in R_f values persisted in all the compounds tested. Thus although the intermediate activity peak has not received positive chemical identification there is some justification for confidence that the ^{14}C activity found here is incorporated in the structures of proline or alanine.

The initial activity peak found in the chromatograms is of even more questionable identity and is likely to consist of more than one substance. The published (Zweig, 1972) R_f value for glucose is 0.22 in n-butanol:acetic acid:water 4:1:2 v/v, (practically identical to 12:3:5), is 0.22. My trials indicate an R_f value close to 0.10 for glucose. The activity which almost always was found at the origin cannot be due to glucose alone. There are many substances which make no significant migration in this solvent including some carbohydrates

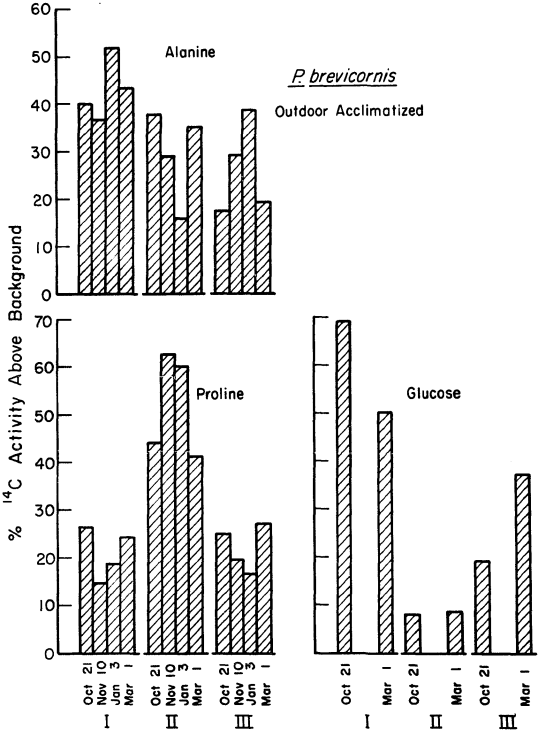
and other classes of compounds also. However it was felt that the usually distinct peak which occurs near the R_f range of 0.10 would be glucose, although no positive identification was made.

The method for estimating amounts of activity at strip sites of activity should be described. First, the raw data were taken from the liquid scintillation ticker tape and recorded as number of disintegrations per minute. These data were transferred to normal graph paper and plotted as percentage of dpm above background against strip position, or R_f. Figure 8 is a plot of percent activity versus R_f for alanine fed beetles, to illustrate how the data appear in this form. To make the bar graphs shown in Figures 9 and 10, the activity present in the individual peaks themselves was calculated. A total integration of the activity peaks was not done but rather 2 or 3 of the highest counts were taken and compiled. Consistency was strived for in this process so that peaks could be fairly compared from different chromatograms.

Seasonal Distribution of ^{14}C in Isotope Fed Insects (Fig. 9)

The glucose fed group provided useable data for only two of the sampling periods, fall and early spring. In fall, a very high percentage of counts above background were found at position one on the chromatogram, indicating that much of the ingested isotope had remained in the form of glucose. At this time less than 20% had been converted to glycerol, where some ^{14}C activity was also present, and very little (less than 10%) was present at position II. By late winter however, about 20% less was found at position I and about 20% more was present in position III, the glycerol spot. The percentage of activity at position II remained low.

Fig. 9 Seasonal distribution of ^{14}C in P. brevicornis hemolymph. Histograms of outdoor, winter acclimatized insects showing percent ^{14}C dpm of sample (ordinate), versus date (abscissa). Insects were originally fed either glucose, proline, or alanine with uniform ^{14}C label. I, II, and III refer to major ^{14}C activity areas on chromatogram.



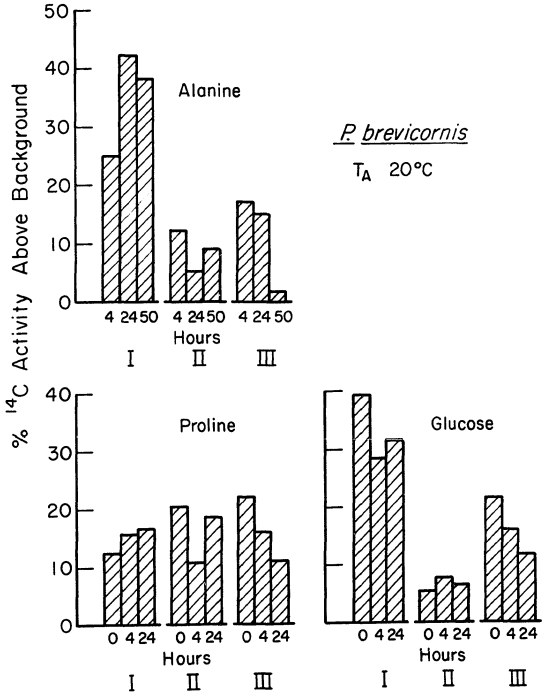
The percent of activity in the alanine fed group remained near 40% at position I except for the January 1 period when 51.5% was recorded here. There is a stepwise decline in activity at position II over the fall and winter until March 1. Percent activity increased to 35% by March 1 from a value of 15% in January. An almost mirror image increase in the percent of activity located in glycerol (Position III) was seen through January, and then declined by March when ^{14}C activity approached the level recorded in early fall.

The pattern in the proline fed group was quite different from that of the alanine fed group. The percentage of activity was smaller at position I throughout the fall and winter. A drop in activity at this position after October 21 was followed by a small increase through the winter. At position II a much higher percentage of activity was found when compared with alanine. After October 21 the percentage increased and remained high until late winter when a drop in activity was observed. At position III, the glycerol area, activity dropped slowly after October 21, and remained low until late winter when the level rose again by March 1.

Distribution of ^{14}C after Sudden Rewarming (Fig. 10)

The chromatographic pattern of ^{14}C isotope distribution was also studied in an experiment involving sudden deacclimation of winter hardy beetles. This experiment was conducted in late March. Beetles from each isotope fed group were taken to the lab where, at time intervals after removal from the cold, hemolymph samples were taken, chromatographed, and counted in the manner described above. Figure 10 shows the results

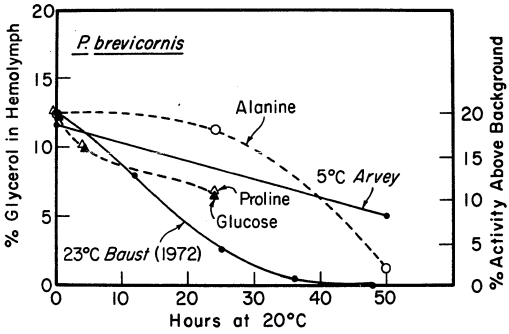
Fig. 10 Distribution of ^{14}C after short term reacclimation of ^{14}C fed insects. (Fed as labeled glucose, proline or alanine). Histograms of ^{14}C activity from chromatographed hemolymph of winter acclimatized P. brevicornis rewarmed to 20°C. Ordinate: percent ^{14}C dpm of sample above background. Abscissa: time in hours since rewarming. I, II, and III refer to major ^{14}C activity areas of chromatogram.



of this trial. Although the data point for time zero was lost in the alanine fed beetles, percent activity at position I tended to rise after exposure of several hours to a warm environment, whereas percent activity at position II appeared to decline quite markedly over the 50 hour test period. At position III where glycerol was found, percent activity rose initially within the first 4 hours of rewarming, then declined drastically until 50 hours, when activity approached zero in this fraction.

In the proline fed group the only apparent significant change in activity distribution occurred after 24 hours at position III where glycerol is found. In contrast to alanine and glucose fed groups however chromatograms of proline fed animals showed higher activity levels at position II in comparison with position I, whereas alanine and glucose fed groups have higher percentages of activity at position I. Rewarming or reacclimating winter acclimatized insects results in loss of hemolymph glycerol content. The rate of loss depends upon the temperature of reacclimation. Figure 11 shows rate of loss at both 5°C and 23°C (Baust, 1972) of glycerol content from hemolymph, and the rate of loss of ^{14}C from the glycerol moiety of alanine and proline fed groups at 23°C. This was done to compare the rate of ^{14}C loss from glycerol with the loss of glycerol itself during the rewarming period. The rate of ^{14}C loss from the glucose and proline fed was approximately the same and appears similar, at least initially, to the rate of glycerol loss at 23°C from the data of Baust (1972). ^{14}C loss rate from glycerol of the alanine fed group however was low

Fig. 11 Rate of glycerol loss at 5°C and 23°C compared to rate of ^{14}C loss from isotope fed P. brevicornis at 20°C.



initially but increased greatly after 24 hours or so.

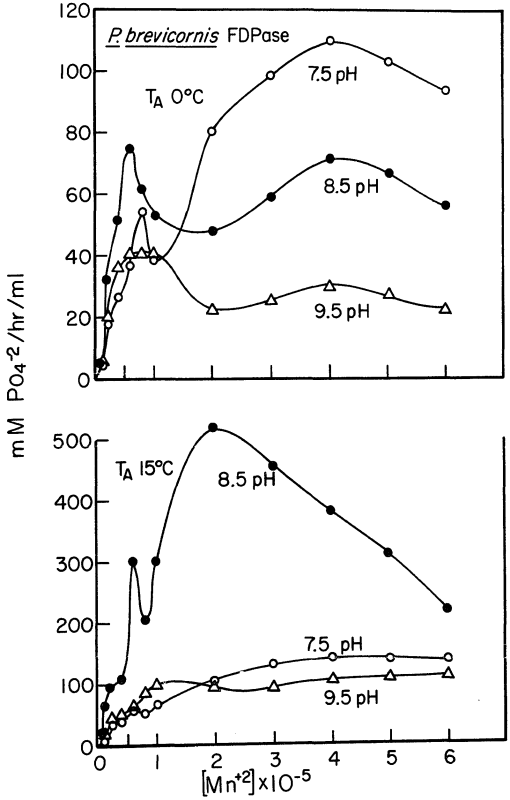
The implications of these results will be discussed in a following section.

Enzyme studies

Laboratory studies were initiated to attempt to assay the relative activity of 2 enzymes, fructose diphosphatase (FDPase) and α-glycerol-phosphatase (α-Gpase). The FDPase activity profiles presented in Figure 12 represent the amount of inorganic phosphate (Pi) released per hour of incubation per milliliter of enzyme preparation. The preparation of the enzyme has been described under methods. The amount of (Pi) released is measured against the concentration of inorganic cofactor, either manganese, Mn^{+2} or magnesium, Mg^{+2}. Mn^{+2} was found to be the best cofactor, so Mg^{+2} was not used. Figure 12 is FDPase activity, using Mn^{+2} cofactor at 0°C. At this temperature, the greatest activity occured at pH 7.5 with a Mn^{+2} concentration of 4×10^{-4}M. The curve of activity for all three pH's was not smooth and continuous but interrupted by smaller peaks and valleys in the lower range of Mn^{+2} concentrations. For example, at pH 7.5 there was a peak at 8×10^{-5}M Mn^{+2}, and then a drop in activity before the largest peak at 4×10^{-4}M Mn^{+2}. At pH 8.5, a sharp initial rise in activity was seen until 6×10^{-5}M Mn^{+2} was reached, then a gradual drop followed by another rise until the final peak activity was reached at 4×10^{-4}M Mn^{+2}. At pH 9.5 a similar sharp rise occurred initially, then a drop with another slight increase at 4×10^{-4}M Mn^{+2}.

Figure 12 also shows activity of FDPase when incubated with the

Fig. 12 FDPase activity with varying Mn^{+2} cofactor concentration. Activity in millimoles of inorganic phosphate released per hour per milliliter of enzyme preparation. Top figure: 0°C; bottom figure: 15°C.



same cofactor at a higher temperature (15°C). In this case maximum activity was at pH 8.5 at a cofactor concentration of 2×10^{-4}M Mn^{+2}. Very little increase in activity was apparent at pH 7.5 and 9.5 at the elevated temperature.

An attempt was made to assay the activity of α-glycerolphosphatase, an enzyme which catalyzes the final hydrolysis of inorganic phosphate from α-glycerolphosphate to yield glycerol. Under all the conditions which were examined, no significant amount of activity could be detected from this enzyme. Various combinations of temperature, pH, cofactor and substrate concentration were tried without success.

Microhabitat Winter Temperature Measurements

Figure 13 is compilation of the temperature data obtained from a natural stump which was wired with thermocouples. Two such stumps were prepared but data from only 1 was included in Figure 13. Included are maximum and minimum air temperature data taken throughout the winter at the same locality. Temperatures were already below freezing when the first stump was wired in early October. Three of the original 5 leads ceased functioning. Number 2, the lead next to the outside never functioned, number 5, the most interior, quit in early November, and number 1, the most exterior lead, quit in mid January.

The second stump was providing temperature data by November 3. Lead number 6, the innermost, ceased after one day of operation in November, number 2 quit in mid December, and number 5 in early January. The remaining 3 functioned throughout the season. For comparison, the pattern of snowfall during the first part of the season is included in

Fig. 13 Microclimate of P. brevicornis, 1970-71. Location: 40 mile Steese Highway. Stump temperatures at different depths in stump, air temperatures and snowfall depths at site.

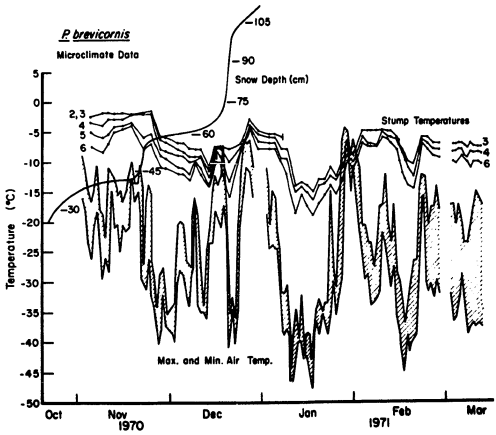


Figure 13. This does not represent actual snow pack thickness on the ground, or on the stumps, only the snowfall pattern with theoretical cumulative thickness. In practice, due to settling, some drifting, and changes in subsurface snow structure the actual amount of snow laying on the ground at any time does not attain the amounts pictured in Figure 13. (See Figure 1 for actual snow accumulation over the stump). This information can be used as an indication of the time course of the snow pack accumulation, especially when the periods of heaviest showfall occurred. The data are not continued after January since very small amounts fell after that time and since by then the snowpack had reached sufficient depth to provide insulation around the stump. At this time some 30 cm of snow (12 inches) covered the tops of the implanted stumps. It is evident that the most deeply implanted leads fluctuated least in temperature and remained several degrees in temperature above the outer leads throughout the season. The effect of the snowpack in damping thermal osccillations was indicated by the fact that wide variations in air temperature appeared to cause short term fluctuations in temperature readings early in the season, prior to January 1, before the great increase occured in snow depth.

The minimum temperature experienced by any of the leads was -19°C and occurred on January 17 when air temperatures had dipped to below -40°C for about 5 days. At that time, one of the interior leads on this stump experienced a temperature of -15°C. This occurred in the third lead from the interior, and although the data are lost, it is improbable that the inner most readings reached much below -10°C, a

moderate cold temperature, when the extreme low air temperature is considered.

Also to be noted is the lag in stump temperatures of several days although air temperatures varied in the extreme in a matter of a few hours. Thermal inertia becomes more pronounced as the snow pack deepends later in the season.

Personal observations while collecting insects in the field indicated that P. brevicornis was generally found during the winter in the punky layers of the stump, usually within six inches or so of the exterior bark or moss. They were not seen inside the solid interior cores of the stumps. In stumps which were decayed throughout. the innermost portions of the stump contained very high moisture contents where almost no adults were found. P. brevicornis seemed to aggregate in the dryer, channeled, outer layers.

DISCUSSION

Glycerol Accumulation, Supercooling, Diapause and Hibernation

Winter accumulation of glycerol in insects has prompted many investigations. It is becoming possible with certain insects to predict conditions under which glycerol accumulation will occur, and often, the quantity and rate of formation. Techniques are now available to trace many of the biosynthetic steps involved in glycerol formation. However many questions remain unsettled in the area of winter hibernation and cold hardening. One concerns the mechanism triggering the start of glycerol production and hibernation. Environmental factors such as decreasing temperatures and photoperiod may act directly upon appropriate organs to produce the required changes at the molecular level. It is also possible that the metabolic machinery of overwintering insects is linked to the environment by internal rhythms such as is known for other organisms and other natural processes including diapause. Little work has focused on this aspect of the seasonal cycle, and although many workers report that glycerol accumulation can be induced by lower acclimation temperatures even during warm months, there are indications by some workers, e.g. Baust and Miller (1970), that when the normal winter regime of cold temperatures is broken, a rapid conversion of glycerol into other metabolites occurs. Once lost, glycerol may not be resynthesized, a result which was found also in the present work. This suggests the irreversible nature of at least part of the overwinter sequence in P. brevicornis, although resynthesis of glycerol after its disappearance is not unknown (Dubach et al., 1959).

The situation is perhaps analogous to certain features of mammalian hibernation where the length of time spent in any particular phase of hibernation can be predicted by the ambient temperature at which the animal is hibernating. Twente and Twente (1970) working with Citellus ground squirrels have shown that higher temperatures apparently act to speed up the biological clock, resulting in less time spent in any given phase of the hibernation cycle. Internal timing of adult insect hibernation, including glycerol synthesis remains a possibility in my view. Beck (1968), Adkisson (1966), Saunders (1970, 1971) and others have adequately shown control by circadian oscillation and photoperiod over diapause in insects. It seems that extreme temperatures can override the effects of photoperiod (Beck, ibid.; Saunders, 1971; Mansingh and Smallman, 1971). P. brevicornis and other arctic insects may spend more than six months of the year in a situation where temperature is the primary environmental limiting factor. Thus photoperiod may be responsible for the control of events leading up to hibernation, in other words, there may be internal rhythms which are entrained to the light-dark cycle; once freezing temperatures and snow cover prevail, temperature must be the controlling factor for all metabolic reactions.

Baust and Miller (1970, 1972) reported temperature dependent accumulation of glycerol in P. brevicornis, where midwinter titers of glycerol varied directly with ambient temperature, even over short time spans. Hemolymph supercooling points were also shown to decrease at lower temperatures. In contrast, my data indicate that

glycerol levels tend to build during the fall, and to more or less stabilize for the remainder of the winter until warmer spring temperatures prevail. I could demonstrate no short term, temperature dependent fluctuations at subfreezing temperatures until spring approached. At that time glycerol concentrations decreased slowly over a 30 day period until actual thawing and snow melt ensued. There is little pertinent literature to review on this point, because most workers have concentrated more on experimental and theoretical aspects of cold hardiness than on observations of seasonal aspects of glycerol accumulation. Some of the studies that have touched upon this problem include those of Krunic and Salt (1971) and Nordin et al. (1970). Krunic and Salt (1971) followed the seasonal changes of glycerol levels and supercooling points in 2 species of Megachile (Hymenoptera) prepupae, one exotic, the other indigenous, in Southern Alberta. Neither species was freezing tolerant, although the indigenous species could successfully overwinter and exhibited greater glycerol accumulation and lower supercooling points than did the introduced species. A maximum glycerol level of 5% of fresh weight was reached by the indigenous species by December, which then fell gradually until the spring approached and values reached nil. Once lost, glycerol was not regenerated by incubation at lower temperatures. The mechanism whereby glycerol accumulation is initiated was not studied.

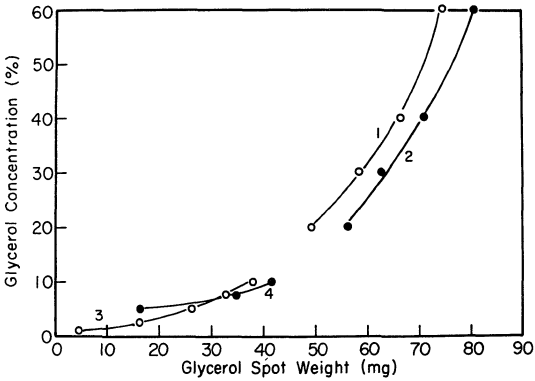
Nordin et al. (1970) looked briefly at the temperature relationships of glycerol accumulation in the carpenter ant Camponotus pennsylvannicus, a species in which adults overwinter. They found that although the ants

were not freezing tolerant, glycerol accumulated when they were held at temperatures ranging from +4°C to -3°C. Judging their data, there appears to be a temperature optimum for this process spanning 5 to 6°C (+3 or 4°C to -1 or -2°C). Below -3°C glycerol accumulation was inhibited, and at -6°C all the ants died. Approximate maximum concentrations of 2% of live body weight were attained, which, if expressed as concentration in hemolymph or water fraction only, assuming the water fraction of the total body weight is about 55% (Dubach et al., 1959), is approximately 4% glycerol. This is comparable to many of my values taken from overwintering, freezing tolerant P. brevicornis. It is obvious that while some insects are freezing tolerant with given concentrations of glycerol, others are not necessarily so, a fact which has been frequently pointed out by other workers (Sømme, 1964; Tanno, 1962; Salt, 1957; and Asahina, 1966). The converse is also true, where insects which cannot be shown to possess glycerol nevertheless exhibit tolerance to freezing temperatures and even to actual freezing.

Reported values for glycerol concentrations of overwintering insects in all stages of development are extremely variable and difficult to interpret. The variance may be due in part to 3 or more factors. The assay technique used may be one of the most important sources of variability. The chromatographic technique described by Perkins and Aronoff (1959), has been modified and used by Sømme (1964), Baust and Miller (1970), among others. The insect material to be spotted on the chromatograph paper may be prepared in various ways. Hemolymph may be extracted by syringe and spotted without further preparation or handling,

which was done in the studies by Baust and Miller (1970), and in part of this work, or glycerol can be extracted from whole insect homogenates, recombined with an appropriate amount of solvent, and then spotted. Errors are magnified in even the simplest procedure when dealing with specimens which weigh less than 10 mg as does P. brevicornis. In the present study for example, hemolymph was extracted from individual restrained insects with a 1 microliter syringe by direct puncture of the cuticle. Pooling hemolymph from 2-5 individuals was usually necessary to obtain a full microliter sample, but introduces a possible source of individual variation into the problem. Extreme care was exercised to prevent the suction of air into the syringe which could then cause inaccurate volumes. Visualization, cutting, and weighing of the spots were also sources of error. It was found that standards had to be run with each sample, and separate, individual, quantitative curves had to be constructed, because a given quantity or concentration of glycerol could yield different results with individual sheets of chromatographic paper. In this connection, Baust (1970) reported a linear correlation for spot weight and glycerol concentrations from 2-60%. I was unable to duplicate this finding. My work (Fig. 14) indicates that the curve for glycerol of standard concentration versus spot size is nonlinear. The curve has the general form of a logarithmic function, i.e., $y=x^2$ where y=concentration, x=spot size, a result which conforms to the principle that area is not a linear function of concentration, which is a linear measurement. This may account for the fact that Baust (1970) and Baust and Miller (1971) reported significantly

Fig. 14 Standard curves of glycerol concentration versus spot weight. Solvent: n-butanol:acetic acid:water (12:3:5). Paper: Whatman #1. Time: 18 hours. Four separate chromatographs are represented.



larger maximum values for glycerol concentration in P. brevicornis than I did. The greatest concentration that was detected during winter of 1971 was 12.5% whereas Baust (1971) reported values of over 20%. I feel that my values are trustworthy, especially because an alternate method of glycerol measurement, a gas-chromatographic procedure, produced very similar results to those obtained using paper chromatography. Therefore, until the reasons for the discrepancy between our respective data can be determined, I will refer to my own values for glycerol levels.

Asahina (1966) summarizes some of the reported values of glycerol accumulation in 26 species of insects, as well as the lowest freezing temperatures which could be tolerated for a 24 hour period. From this it can be seen that freezing tolerance may not be correlated with glycerol accumulation in many insects. Dendroctonus monticolae larvae containing 23.4% glycerol in the water fraction had no tolerance to freezing temperatures (Sømme, 1964), whereas Hyalophora cecropia pupae containing 2.8% glycerol can survive -70°C (Asahina, 1966, unpub.). Values range from 0% to 25% of fresh weight in Bracon cephi larvae (Salt, 1959), an extremely high concentration when one considers that if viewed as a percent concentration of the water fraction only, the value would be over 40%. Thus it seems very tenuous to attempt to generalize about glycerol accumulation in insects, both from the standpoint of maximum accumulation values to be expected in a given climatic area and interpreting the degree to which cold and freezing are imparted to the insect by given levels of glycerol.

Another reason for variance in reported values of glycerol accumulation comes from the fact that different orders and species are studied, naturally, as well as different life stages. Thus, for example Chino (1957, 1958, 1960, 1961) studied eggs of the Bombyx silkworm, Salt (1957) studied larvae of Bracon cephi, Takehara (1966) studied prepupae of Monema flavescens, Sømme and Velle (1965) studied pupae of Pieris brassicae.

Another, perhaps critical, source of variation in these studies is the actual handling and care of specimens. As Baust and Miller (1972) point out: "specimen handling, especially with respect to temperature exposure, is a critical factor and the complete thermal history of insects used in studies of freezing resistance must be known." Rates of change of both physiological and biochemical parameters in insects can be quite rapid, and significant differences can be detected in just a few hours time, as shown in Figures 3, 6, and 10.

Microenvironment of Overwintering P. brevicornis

Baust (1970) showed that the common overwintering habitat for P. brevicornis in the Fairbanks area, i.e. decaying stumps, is quite stable with respect to thermal fluctuations in the environment, and that this stability was progressively greater as one proceeds from the outside layers of wood to the interior of the stump or tree. His measurements were carried out on isolated, detached stumps subjected to laboratory temperature fluctuations. In the present study it became feasible to monitor real stumps on a daily basis almost continuously throughout one winter season with the aid of thermocouples which were implanted

at different depths in the wood. Readings taken in this manner allowed direct measurements to be taken on the winter microhabitat without disturbance of the stump or surrounding snow cover. Thus the effect of a natural snow cover on microhabitat temperatures could be determined.

The original assumption was that, under a normal season's snow cover (.25-.50M), and within natural decayed stumps, the overwintering microhabitat of P. brevicornis could be quite moderate with regard to both temperature fluctuation and temperature extremes. The data presented in Figure 12 quite clearly show that this is correct. Short term temperature fluctuations were effectively damped in amplitude especially within the deeper wood layers. Also extreme low environmental temperatures were not attained beneath the snow layer, where the lowest temperature found approached only -20°C, even in the outermost shell of the decayed stumps. The implications carried by these findings for hibernating insects is clear. It could be assumed that animals hibernating in deeper levels of the forest moss, litter, or soil layers would accrue similar thermal advantages.

It is interesting that although P. brevicornis can withstand freezing to extremely low temperatures (-40°C to -80°C) without apparent subsequent harm, the normal winter thermal regime in interior Alaska is such that tolerance to temperatures in this range is not normally required, at least in the preferred microhabitat. However, climatic conditions at high latitudes are so extremely variable from year to year that in a season marked by late or little snowfall, combined with early

(November) low temperatures, adaptation to these extremes may indeed be advantageous. Also, since the relative energy costs to the individual insect in terms of becoming frost resistant are not clear at the present time, it could be that extreme frost resistance is little more costly energetically than a moderate degree of frost resistance.

Seasonal Lipid Changes

Total lipid content measurements could not be made with the regularity and precision required for a careful seasonal analysis. The data from some twenty samples presented in Figure 7 are not sufficient to describe any marked seasonal trends. The main difficulty found in total lipid measurement was that of obtaining sufficient numbers of beetles to comprise measureable fat samples. It was found that 100 insects were barely enough to carry out the procedure without incurring the risk of committing gross errors in measurement. The supply of beetles was limited and therefore sampling was not frequent enough to provide a clear picture of the lipid situation. The values obtained tended to cluster around 20% of dry body weight except possibly for the winter months where my data show a decrease in lipid levels. Kaufmann (1971) discussed the relative size of the fat body in P. brevicornis over the fall, winter, and spring months. It was her impression that the size of the organ was greatest at the onset of hibernation, then decreased until late winter when feeding had apparently begun. It is possible that during the months when oviposition is taking place, fat stores could become greatly depleted. However no significant depletion during the period of active glycerol accumulation is evident.

However it must be kept in mind that the fat body in insects has many functions besides that of regulating fat metabolism (Kilby, 1963). Salt (1959) made cursory examinations of fat levels in cold hardened and non-cold hardened larvae of Bracon cephi. He found levels of 42% (dry weight) present in non-hardy larvae, compared with 38% in the cold hardy group. This does not indicate a large scale conversion of lipid stores into the glycerol pool. Chino (1957) did not find significant reductions of lipid stores in the eggs of Bombyx during the time of glycerol accumulation. He observed that fat content slowly declined throughout the diapause stage, whereas carbohydrate content decreased rapidly and dramatically. Furthermore he discovered that anaerobiosis has no effect on the rate of glycogen dissappearance into sorbitol and glycerol, but greatly retards the resynthesis of glycogen after diapause is broken. His hypothesis regarding glycerol accumulation in diapause involves first a blockage of normal oxidative metabolism at the level of the cytochrome system. This means that molecular oxygen is not the final acceptor of hydrogen produced by the oxidation of fats, glycogen, and perhaps other substrates. The acceptor for this hydrogen (in the form of reduced NAD^+) may be triose phosphate which could then be converted to glycerol. Chino (ibid.) did not consider fat to be the major contributor of the three carbon units necessary to synthesize glycerol and sorbitol but he did assume that normal metabolic needs during diapause were met by partial oxidation of fats, yielding hydrogen. This is consistent with the general finding that lipid levels decrease slowly and moderately during diapause or hibernation.

Fatty Acids

As noted previously, this has not been an exhaustive study of fatty acids in P. brevicornis, and as a consequence most of the quantitatively less important acids are considered only to the extent of their tentative identification in the samples. More precise estimates of the eleven more abundant fatty acids are given in Table II.

Observations of House et al. (1958) that fatty acid composition varies with environmental or experimental rearing temperatures in insects are supported by the results of this study. Desaturation tends to occur in the winter months, saturation in the summer and at higher acclimation temperatures. The major acids of P. brevicornis are palmitic (16:0), palmitoleic (16:1), oleic (18:1), and linoleic (18:2), but not in that order. The predominance of these acids has also been reported by Barlow (1964) on 2 other species of carabid beetles, Calosoma calidum and Harpalus caliginosus. It appears that these acids may not assume the same importance in other insect groups and that other acids, for example stearic (18:0) or linolenic (18:3) may be more important (Fast, 1964). In the present study one can note from Table I a seasonal shift in the abundance of saturated versus unsaturated or less saturated fatty acids. Buffington and Zar (1968) found an increase in levels of unsaturated fatty acids during the winter hibernation of Culex pipiens and suggested as does Barlow (1964) that unsaturates could be mobilized more readily during cold stress because of their lower melting points. Harwood and Takata (1965) noted accumulation of unsaturated fatty acids when Culex tarsalis was exposed to temperatures and photoperiods

which normally induce hibernation. Lambremont et al. (1964), and Toombes (1966), reported decreases in the amount of mono-unsaturated fatty acids, and slight increases in polyunsaturates, in 2 species of curculionid beetles. Toombes (ibid.) thought that these changes were associated with aestivation and not cold stress itself, and suggested that the changes in saturation are associated with diapause and not related to thermal stress. Pantyukhov (1964) noted increased levels of unsaturated fatty acids in different populations of the brown-tail moth, Euproctis chrysorrhoea, and the gypsy moth, Lymantria dispar, with the northern, cold hardy populations showing a greater increase in unsaturation during cold periods than the southern counterparts.

It is becoming increasingly likely that cellular membranes will be implicated as the primary site of freezing injury. Mazur (1970) in a review of freezing in biological systems concludes that membranes are much more susceptible than soluble enzymes to freezing injury in cells. He states (ibid., p. 947):

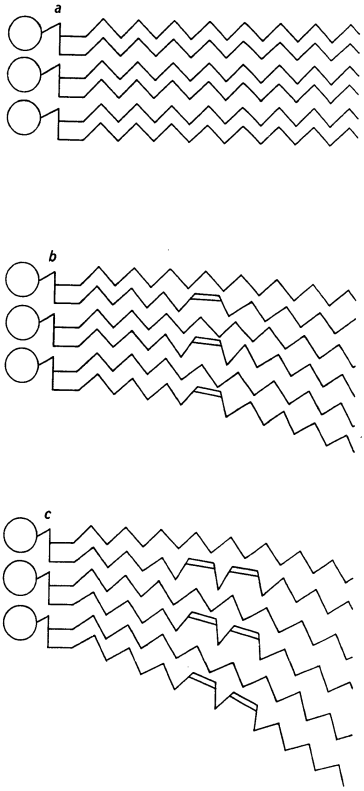
> "Furthermore, cells that are injured either by solution effects or by intracellular freezing undergo damage to surface and internal membranes, and the damage from the solution effects can be reduced by the addition of certain additives that appear to act at the cell surface."

It is known that the process of frost hardening in plants involves increases in the permeability of the cells to water (Levitt, 1966; Mazur, 1969). This is probably due to the fact that freezing initially occurs outside the cell, creating osmotic pressures across the membrane which can only be equalized by the passage of water from the cell.

The structure of cell membranes is known to consist of a bilipid

layer which provides the gross structural properties of the membrane, with proteins imbedded in and extending through the inner and outer membrane surface, or attached to one surface or one side of the phospholipid layer only, depending on the function of the protein (Fox, 1972). The lipid layers are so oriented that the outer and inner membrane surfaces consist of hydrophilic, polar phospholipids which are attached to one end position on the glycerol molecule. The remaining two positions on the glycerol molecule are occupied by internally oriented fatty acids which are non-polar, and hydrophobic. Thus both inner and outer membrane surfaces contain hydrophilic polar heads, with the interior of the membrane consisting of the non-polar hydrophobic hydrocarbon chains. When the fatty acids comprising the membrane are saturated, the result is that the fatty acid tails of the membrane interior tend to align in a tightly packed crystal-like configuration, which tends to inhibit the rate of transport of materials such as water through the membrane. A high proportion of unsaturated fatty acids in the membrane on the other hand, causes the crystalline alignment to be disrupted by the presence of the double bonds, which make the membrane structure more fluid in nature, and more amenable to the passage of water. Membranes rich in unsaturated fatty acids allow transmembrane transport to proceed at rates up to 20 times faster than when the lipids contain few unsaturated fatty acids (Fox, 1972; Wilson et al., 1970; Fox, 1969). Figure 15 (from Fox, 1972) illustrates schematically the relationship between structure of the membrane lipid and degree of fatty acid saturation, and depicts how the membrane becomes more fluid with increasing unsaturation in its phospholipids.

Fig. 15 From Fox (1972). Effects of phospholipid saturation on biomembrane fluidity. Circles represent polar, hydrophilic group of phospholipid which attaches to glycerol molecule (vertical lines). Parallel zig-zag lines represent non-polar, hydrophobic fatty acid tails of phospholipid. a) carbons of fatty acids totally saturated with fatty acid chains tightly packed; b) and c) increasing desaturation of fatty acids leading to less tight packing of fatty acids and increasing fluidity of membrane.



The review by Hazel (1973) on temperature-induced alterations in membrane composition indicates that one response of almost all poikilothermic organisms to low environmental temperatures is a decrease in the degree of saturation of their fatty acids. To quote Hazel (1973):

> "In summary, either cold acclimation or habituation of a chronically cold environment is invariably associated with an increased degree of unsaturation of the fatty acid moieties of phospholipids from a wide variety of species at all levels of biological organization..."

The consequences of saturation changes in membranes are such that permeability functions are affected by the relative liquid or crystalline nature of its constituent phospholipids, which is dependent upon their saturation. It has been shown that there is a definite transition temperature at which the membrane changes phase from liquid to crystalline and vice versa, and this transition temperature depends upon saturation in the phospholipids (Steim et al., 1969).

Others have shown that transport rates across the cell membrane are dependent upon phase characteristics. For example, Overath (1970), in a study of phase transitions of different membrane lipids at different temperatures, obtained results which indicated that both membrane transport and function depend upon mobility of the constituent lipids which in turn are correlated with the degree of packing of the hydrocarbon chains.

Therefore, it seems reasonable to suspect that the changes noted in fatty acid saturation in P. brevicornis at different seasons and at different temperatures play a distinct role in their ability to

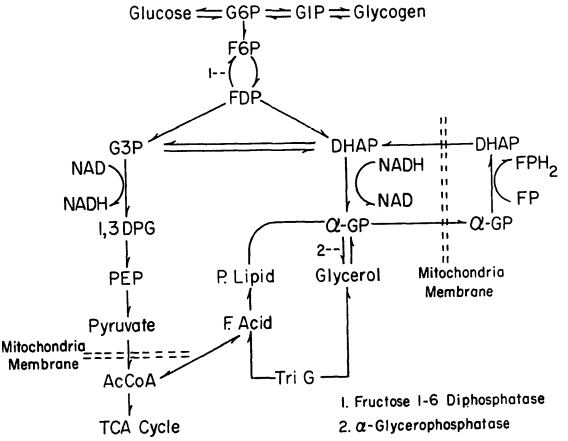
survive freezing. If the major protective action of glycerol and other cryoprotective additives is indeed at cell surfaces then the combined effects of glycerol and fatty acid saturation changes may act in concert to assure proper transport across cell membranes.

One intriguing aspect of the metabolism of lipids and phospholipids with regard to this study is that glycerol and its derivatives α-glycerophosphate and dihydroxyacetone phosphate are important intermediates in synthesis and degradation (see Fig. 16). Kennedy (1961) was one of the first to elucidate many of the relationships in phospholipid metabolism, and Gilbert (1967) following his work reviews the situation in insects. α-glycerolphosphate occupies a key position since it can be converted to phosphatidic acid which in turn can enter into synthetic pathways for either glycerides or phospholipids. Gilbert (1967) presents evidence which shows that free glycerol itself may give rise to α-glycerophosphate, thus playing an important role in lipid metabolism. Large hemolymph titers of free glycerol found in many diapausing and hibernating insect species may be more related to the needs for lipid and phospholipid precursors during the developmental periods which commence upon termination of dormancy as well as to any cryoprotectant functions.

It seems a definite possibility that glycerol could function as an intermediate in lipid metabolism during periods of cold acclimatization. It is during this time that important changes in fat composition and fatty acid saturation take place. Perhaps the most important adjustment to cold by insects involves changes in the lipids of cell membranes rather than the accumulation of a cryoprotectant which in itself can

Fig. 16 Schematic representation of metabolism in insects, showing possible interrelationships of glycerol, lipids, phospholipids, and carbohydrates. Enzymes named are those studied here.

Insect Intermediary Metabolism



impart protection to cell membranes and contents.

It must be pointed out that the analytic techniques utilized in this study were not favorable for the most efficient extraction of phospholipid, the main lipid component of membranes. Petroleum ether used as the extraction solvent is less suited for phospholipid extraction than chloroform/methanol (2:1 v/v) because petroleum ether is a nonpolar solvent, and phospholipids contain polar subunits. Therefore the extent to which the saturation changes noted above reflect changes in the membrane components as opposed to neutral lipids is not clear. Further, more specific analyses of phospholipid changes would be necessary to clarify this question. It seems most likely at this point to identify the saturation changes with the neutral lipid fraction because of the method utilized in extraction.

Amino Acids

Levels of free amino acids in insect blood have invariably been found to be high, reaching much greater concentrations than those found in vertebrates for example (see Sacktor, 1965). Free amino acids are often utilized as a direct energy source, at least in the initial phases of muscle work (Sacktor, 1965; Sacktor and Childress, 1967; Lue and Dixon, 1967; Weis-Fogh, 1967; Barker and Lehner, 1972). Proline is an especially abundant hemolymph free amino acid and has been shown by Bursell (1963), Sacktor and Childress (1967), and Barker and Lehner (1972) to be able to penetrate mitochondrial membranes and enter the citric acid cycle through reversible transformations to glutamate and α-ketoglutarate in at least some insects. It is probable that such

biochemical pathways are utilized in the majority of insects.

My results indicate that amino acid concentrations are generally high in P. brevicornis, ranging in value from 0.159 to 1.20 M for concentration. Proline is by far the major free amino acid in P. brevicornis comprising from 39.5 to 67.0% of the total number of micromoles of amino acids present in the samples. Florkin and Jeuniaux (1964) report that proline and glutamic acid are in general the most abundant amino acids present in insects. The data in this study are not sufficient to allow us to speculate on seasonal trends in amino acid concentration except to note that the highest total amounts tend to occur in the fall and winter months while the lowest occur in summer months. More systematic sampling procedures would be required to elucidate these trends, and unfortunately the time and facilities available for this work were not sufficient for that.

Increased winter concentrations of free amino acids would seem advantageous for the insect from the standpoint that more molecules would be present to influence colligative properties, such as freezing and supercooling points. However neither of these parameters is particularly low in winter in P. brevicornis, although they are significantly lower than in summer (see Baust and Miller, 1972). To be really effective in lowering supercooling and freezing points, the concentrations of amino acids would probably have to be so great as to reach toxic levels in the hemolymph, which would obviously be a detriment.

Although little difference was found between levels of amino

acids found in non-hydrolyzed and hydrolyzed hemolymph, the possibility still exists that seasonal differences may be found in hemolymph levels of peptides or other nitrogenous materials besides amino acids. Baust and Naumann (1969) carried out brief experiments in P. brevicornis to test this possibility and found no difference between hydrolyzed and non-hydrolyzed hemolymph at least at the particular time of year in which their work was done. Florkin and Jeuniaux (1964) find little evidence except in a few isolated cases, for significant non-amino acid, nitrogenous compounds in insect hemolymph.

Possible transformations of certain amino acids into the glycerol pool will be discussed in a later section.

Glycogen Metabolism

Insect glycogens have not been reported to differ chemically in any significant way from other animal glycogens; for example, Stevenson and Wyatt (1964) found that they serve satisfactorily as a substrate for phosphorylase enzymes. Glycogen has long been known to be abundant in insect tissues, especially in larval stages of the life cycle. Almost all tissues contain glycogen deposits but the greatest amounts are found in the fat body where it is readily extractable with cold TCA. Muscle and other tissues contain some readily extractable, or "free" glycogen, in addition to more or less constant fraction of "fixed" glycogen, which is resistant to extraction according to Kubista and Bartos (1960). Fixed glycogen has not been chemically identified as such and may include other protein-bound polysaccharides (Wyatt, 1967).

In the present study the assumption is made that since identical

extraction procedures were followed with each sample, relatively similar amounts of free versus fixed glycogen were extracted and that final results would not differ only because of variable quantities of fixed glycogen occurring in the extract.

Peak values for glycogen reported in the literature on a fresh weight, whole body extract basis, before the onset of diapause, glycerol formation, or cold hardening depend on the life stage being studied. For example, Chino (1957, 1958) and Takehara (1966) observed glycogen levels in excess of 40 mg/gm, whereas in the present study and in that of Nordin et al. (1970) peak values were usually well below 20 mg/gm. However the former studies were conducted with diapause eggs, Chino (ibid), and prepupae, in the work of Takehara (ibid). Adult insects tend to contain a greater proportion of metabolically inactive support material such as chitin that adds to body weight but reduces the proportion of other fractions relative to body weight. It seems likely that if glycogen content were compared to the water fraction, the values would appear to be more equal over a greater spectrum of insect types.

Interconversion of glycogen and glycerol in insects has been reported by Chino (1957, 1958, 1960, 1961) in the diapause egg of the Bombyx silkworm, where glycogen disappearance was associated with the onset of diapause and the synthesis of glycerol and sorbitol. Reappearance of glycogen and decreased polyhydric alcohol levels occurred upon diapause termination. Chino (1958) demonstrated that the restoration of glycogen content in the eggs was directly linked to diapause termination and not to post-diapause development since the

phenomenon occurred even when embryogenesis was suppressed after diapause by chilling at 5°C. At the onset of diapause, glycogen content in Bombyx eggs was approximately 40 mg/gm, and after 30-40 days in diapause at a temperature of 5°C, dropped to about 7 mg/gm. At the same time polyhydric alcohol content increased from near zero to 31 mg/gm of eggs. Chino (ibid) believed that the total polyhydric alcohol content was less than expected to completely account for the decrease in total carbohydrate reserves because some of the carbohydrate went into the formation of lactic acid during this period, although his analysis showed only traces of lactate during diapause. After diapause was terminated on about the 60th day, glycogen levels increased even though the eggs remained at 5°C. On the hundreth day of chilling, the eggs were transferred to 25°C, and the glycogen content increased to a maximum of 33 mg/gm, at which time polyhydric alcohol content approached zero. Interestingly, glycogen did not increase to levels prevailing before diapause, but declined immediately after embryogenesis began, which indicated that carbohydrate was being used as fuel at this time.

Chino (ibid) further showed that interconversion between fat and glycogen was not taking place. Fat content of eggs during diapause declined slowly, and Chino postulated that fat was being consumed for energy during this time.

Wyatt and Meyer (1959) found a similar conversion in diapausing Hyalophora cecropia pupae, although quantitatively glycogen and glycerol did not reach levels comparable to those reported by Chino (ibid.). However the pattern of conversion with respect to temperature conditions

appears very similar.

Salt (1959), in his study of glycerol accumulation in hibernating larvae of Bracon cephi found no evidence that glycerol and glycogen are interconvertible. However almost no data are presented pertaining to glycogen analysis in the paper. Similarly, Salt (ibid.) states that fat is not converted to glycerol but offers very little data to support his statements.

Takehara (1966) studied glycerol accumulation in overwintering prepupae of the slug caterpillar Monema flavescens and showed that glycogen and glycerol levels vary inversely with respect to each other. Unlike Chino (1958) however, Takehara (ibid) did not find the behavior of glycerol to be directly associated with diapause, but rather that it was dependent upon temperature conditions during diapause. A definite temperature optimum was noted for glycerol accumulation at about 10°C. Glycogen levels increased in the fall, before the rise in glycerol levels. Maximum glycogen values reached about 40 mg/gm in mid-September, after which glycogen declined concurrently with increasing glycerol levels. Glycogen levels fell to and remained near zero for the duration of the period of diapuase. Then the reverse occurred, glycogen rose, glycerol decreased, and after all glycerol disappeared, glycogen began declining again. Baust and Miller (1972) criticize Takehara's (ibid) assumption that glycogen and glycerol are interconvertible because data submitted by Takehara (1966) seem to indicate an impossibly efficient conversion. However Takehara does not propose that his results indicate a strict quantitative conversion because of

individual and seasonal variation of both glycerol and glycogen content. His samples were also sometimes pooled in order to estimate glycerol quantities. Stoichiometric analysis is not feasible under such conditions, and it seems useless to criticize the proposal that glycogen may be converted to glycerol on such grounds. In the present study for instance, the data from whole body, pooled, glycogen extracts, and from pooled glycerol measurements indicates only that there is a likely glycerol-glycogen conversion, but no attempt is made to account for every 3 carbon unit on a gram for gram basis. The fact that at least some isotopic ^{14}C originally administered as glycerol is later counted in the glycogen extracts indicates that indeed, some glycerol can be converted, by some pathway, into glycogen. It does not mean that the glycogen-glycerol relationship is on a one-to-one basis, nor does it rule out the possibility of other substrates entering into the glycerol pool through other pathways. For example, chitin may at times be reabsorbed and converted into glycogen, and perhaps other compounds. Zaluska (1959) showed that a drop in chitin content of Bombyx prepupae was correlated with a rise in glycogen content. Bade and Wyatt (1962) observed the same phenomenon in the Cecropia silkworm. These authors suggest that chitin resorbed from cuticle, in addition to protein and mucopolysaccharide, provides the material for glycogen synthesis after feeding ceases in these insects. Glycogen may also be the source of material needed to make new cuticle at the time of molt (Wyatt, 1967).

Precise, quantitative administration of ^{14}C labeled substances

proved unfeasible because of the small size of P. brevicornis. Attempts were made to inject known amounts of substrate, but this also was impractical for several reasons, mainly because precision was extremely poor. The alternate method of allowing the insects to feed upon isotope treated material, although imprecise, avoided complications associated with other techniques. Cuticle puncture in Pterostichus for the purpose of injection is a traumatic procedure, and results in fluid leakage, rapid desiccation, and often premature death. The major difficulty found in the feeding method described is that unknown amounts of labeled substrate are ingested by the insects, which does not permit quantitative ^{14}C counting. This degree of imprecision was accepted in this study in order to gain a general indication of the direction of carbon flow under special instances. Figure 6 shows that after 48 hours at 5°C, a temperature at which the insects are mobile in winter, glycogen levels have risen from almost zero to about 16 mg/gm of fresh weight. The regression curve (Fig. 3) for glycerol under the same temperature conditions shows that glycerol levels fall from nearly 12% at the beginning to about 5% after 48 hours, giving a 6% loss over this period. This amount lost corresponds to less than 5 mg/gm of glycerol, compared to about 15 mg/gm increase in glycogen. Thus only about one third of the glycogen increase can be accounted for quantitatively from the glycerol conversion. This leaves a remaining two thirds of the increase in the glycogen fraction which must come from various other sources. Although no real information is available as to what these sources might be, there are several possibilities.

Trehalose, the normal blood sugar of insects, or glucose, which is sometimes also found, might at first be considered a likely possibility for a glycogen source. In most inactive states however, concentrations of free reducing sugars are normally quite low, too low to be expected to contribute significantly to any large store of glycogen. In active feeding stages however, significant amounts are sometimes found (Wyatt, 1967), especially in those species with carbohydrate rich diets. Baust (1972), in an attempt to correlate freezing point distribution, acclimation temperature, glycerol levels, and hemolymph trehalose in adult P. brevicornis found trehalose levels of 0.5 gm/% to 5.7 gm/% with little apparent correlation to acclimation temperature.

It has been adequately shown (Wyatt, 1967) that hemolymph sugar levels are regulated in insects as in vertebrates and that trehalose represents a transportable intermediate between storage and utilization sites.

A second possible explanation is that chitin was being mobilized into the glycogen pool. Massive resorption of cuticle in the spring or upon acclimation to higher temperatures seems unlikely because of the short time required for glycogen mobilization. The appropriate enzymes would need to be temperature sensitive and the process of cuticle mobilization would have to begin immediately upon exposure to warmer conditions which would mean extreme temperature dependence of the system. This seems unlikely, especially since no evidence of such changes was detected.

Another possibility would be that the fixed fraction of glycogen

which is normally not readily extractable might somehow be transformed into a more extractable form under these conditions. Although the content of readily extractable glycogen varies greatly from tissue to tissue the fixed glycogen remains relatively more constant. There is some variation however. Kubista and Bartos (1960) found that concentrations of fixed glycogen range from 0.20% in Locusta migratoria pterothorax to 0.09% in the femur. The majority of the total glycogen present is found in such specialized tissues as the fat body where most is of the extractable type. It is also probable that much of the fixed fraction is found in protein-bound polysaccharide complexes, possibly as parts of structural components. Whether those bound forms of glycogen could be released in response to temperature changes is not known, but it remains a possibility.

Amino acid pools may contribute to carbohydrate stores after undergoing deamination. Sacktor and Childress (1967) have proposed from studies of proline metabolism in the flight muscle of the blowfly, Phormia, that isolated mitochondria are able to oxidize proline to glutamate. Proline can penetrate mitochondrial membranes, whereas glutamate and other intermediates of the citric acid cycle cannot. Proline oxidation within mitochondria leads to the formation of citrate, a critical TCA cycle intermediate. With proline thus acting as a primer of the cycle, pyruvate can be oxidized at peak rates upon the initiation of flight, without a delay while the TCA cycle awaits the formation of necessary intermediates. Therefore no pyruvate buildup occurs, though it is formed at a faster rate in glycolysis than it can be

utilized in flight muscle. Sacktor and Childress (1967) suggest that flight muscle mitochondria are deficient in TCA intermediates and that these can be generated from proline oxidation. Barker and Lehner (1972) reported reversible transformations of proline, glutamate, and α-ketoglutarate in the honeybee, Apis. They found that although proline can enter the TCA cycle in Apis, it supplies only a small percentage of the energy utilized for flight, presumably due to the high availability of carbohydrate energy sources in honeybees.

It is possible that under certain conditions amino acids are gluconeogenic, that is, withdrawn from hemolymph or other sources and used not as an oxidizable substrate, but as building blocks for high energy storage material such as glycogen. Whether the amino acids would first be transformed into TCA cycle intermediates as in the above examples, or could be deaminated and enter the glycolytic pathway directly is not clear. However this study provides some evidence that amino acids can be converted to carbohydrates. These data will be discussed in a following section. The extent and importance of this process is not known at present.

Lipids represent another rich organic pool from which materials for glycogen synthesis may be derived. However, as Wyatt (1967) points out, the conversion of lipid to carbohydrate has not been demonstrated in insects, in contrast to microorganisms, plants and possibly helminths (Passey and Fairbairn, 1957), where it has been shown to occur.

Early observations starting with Bataillon and Couvreur (1892), in which glycogen levels in silkworm larvae were shown to increase

even after the cessation of feeding led to the hypothesis that fat was being converted to glycogen during these times. Since the work of Zaluska (1959), and Bade and Wyatt (1962) this phenomenon has been ascribed to the entrance of resorbed larval chitin into the glycogen pool during this period.

In mammalian and most all other systems studied thus far, with the exception of helminths, pyruvate is normally taken to either lactate under anerobic conditions or into the TCA cycle which ultimately yields CO_2 and water under aerobic conditions. The step from phosphoenol-pyruvate to pyruvate is normally thought to be irreversible (Duel and Morehouse, 1946). If this be the case in insects as well, then fatty acids arising from the degradation of lipids would not enter into gluconeogenesis because of the irreversibility of this step.

In this connection, Saz (1969, 1971), Saz and Bueding (1966), Saz and Hubbard (1957), Saz and Lescure (1967, 1969), and Saz and Vidrine (1959), have helped to elucidate a most interesting scheme of anaerobic metabolism and its control mechanisms in Ascaris lumbricoides, a facultatively anaerobic helminth. In these animals, it appears that terminal oxidation is accomplished not by the cytochrome system, but rather by flavins. End products of fermentation in Ascaris are succinate and fatty acids instead of CO_2 and water. Glucose degradation proceeds via glycolysis to phosphoenolpyruvate and from there to oxaloacetate and malate directly by CO_2 fixation. Malate then enters the mitochondria where half is oxidized to succinate and the other half is transformed into pyruvate and CO_2, by oxidative decarboxalation. From pyruvate,

fatty acids can be formed via acetate. Enzyme studies by Saz et al. (ibid.) have shown that the Ascaris system can proceed in the reverse direction to the normal mammalian scheme at certain key points in the pathway. Nothing similar has been demonstrated in insects, even under anaerobic conditions.

A serious drawback to the postulation of a lipid-carbohydrate conversion is the fact that a complete glyoxylate cycle has not been demonstrated in insects (Bade, 1962). This pathway, which has been described only in microorganisms and plants is one in which isocitrate in the TCA cycle is transformed, in the presence of isocitrate lyase into succinate and the two carbon glyoxylate. Glyoxylate then combines with acetyl-CoA to form malate. Malate can then be decarboxylated to phosphoenolpyruvate. Carpenter and Jaworski (1962) detected isocitrate lyase activity in Prodenia pupae and suggested that certain life stages should be more closely examined to determine whether the complete glyoxylate cycle might be operative. This would pertain especially to those stages in which lipid stores are being most rapidly used, and possibly in insects or stages undergoing partial or complete anaerobiosis. Anaerobic conditions may stimulate the conversion of lipid to carbohydrate since in the absence of oxygen, fatty acids are not oxidized (Gilbert, 1967).

Generally, except under special circumstances, like anaerobiosis or the need to rapidly utilize a very abundant lipid source, the conversion of fat to carbohydrate does not appear to be particularly advantageous. As a fuel, fat provides more energy per weight of storage

material because less water of hydration is required in its storage, and on the other hand it provides more metabolic water when oxidized (Weis-Fogh, 1967). Under anaerobic conditions, the carbohydrate being oxidized glycolytically might be preferred as an energy source (Gilbert, 1967). As noted previously, there does not seem to be much evidence for anaerobiosis, either obligate or facultative in hibernating insects. Although Sømme (1966, 1967) and Wilhelm et al. (1961) reported increased glycerol accumulation by exposing diapausing or hibernating insects to anoxia, it remains fairly clear that the oxidative machinery present in active stages remains substantially intact in overwintering insects (Harvey , 1962; Wyatt, 1963), and that anaerobiosis is not a prerequisite for glycerol accumulation. In the present study, exposure of P. brevicornis to 24 hours of anoxia at 30°C did not induce significant increases in glycerol levels, although survival was about 50% at that temperature. At 0°C, survival was 100%, but only traces of glycerol could be detected.

Radioisotope Feeding Experiments

Short term reacclimation

Figure 10 shows that ^{14}C label, originally administered as proline, glucose, or alanine, becomes distributed into at least 3 different compounds or classes of compounds. In itself this may be taken as an indication that mutual transformations from carbohydrate, amino acids, and glycerol are occuring in the insects. In the proline fed group, the percent of the label rose some 4%, from 12.5 to 16.5%, after 24 hours at the origin, where carbohydrates remain in this particular

solvent system. The intermediate chromatograph peak where proline migrates in this solvent showed a net loss of perhaps 2% over a 24 hour period, although a drop of nearly 10% during the first 4 hours of warming was noted. This 10% was apparently partially regained after 24 hours. The percentage of label residing in the glycerol fraction, on the other hand, declined steadily over the sampling period of 24 hours by some 11%. Some of this labeled carbon from glycerol appeared to be incorporated into carbohydrates since carbohydrate showed a net increase in ^{14}C after 24 hours. The reason for the dip in activity after 4 hours in the intermediate, amino acid position, followed by an increase after 24 hours is not clear. It is tempting to speculate that upon initial reacclimation, amino acids, principally proline in this case, and glycerol were converted to carbohydrate. Carbohydrate, then, after a period of initial accumulation began to be catabolized to other substrates, including amino acids to provide oxidizable fuel. This interpretation would be consistent with the general finding that glycerol is converted to carbohydrate, usually glycogen, upon termination of diapause or hibernation.

The pattern of ^{14}C distribution was somewhat similar in the alanine fed group. It should be noted that in this case sampling began after 4 hours instead of at zero hours and ended after 50 hours instead of 24.

The relative amount of ^{14}C found at position I was much greater than in proline. In the period from 4 to 24 hours the percent activitity increased some 17% at this position, then dropped slightly after 24 hours. A smaller amount of ^{14}C was found at position II originally, with the

same pattern of initial decrease occurring after 24 hours, then a subsequent increase after 50 hours as in the case of proline fed insects. Glycerol, position III, again showed a stepwise decline over the sampling period. Interestingly the initial increase at the number I position of 17% cannot be accounted for by the concurrent decrease at either position II or III. The explanation for this descrepancy could come from many factors, probably the most obvious being that new ^{14}C is appearing in this fraction which previously had been incorporated in substances that did not migrate to an area where it was counted in this solvent system.

The hemolymph of glucose fed insects indicates that more ^{14}C remained in glucose, if we assume the first chromatograph peak included the glucose fraction. As acclimation proceeds it appears that glucose was metabolized, lowering the amount of ^{14}C remaining at position I. Relatively smaller amounts of ^{14}C appeared at the intermediate position, in contrast to proline and alanine fed groups, indicating that ^{14}C administered as glucose was not transformed or did not accumulate to a great extent in amino acid compounds. Glycerol again showed a sharp decline as acclimation proceeded, although the ^{14}C leaving this fraction did not appear as a net gain in the other fractions. The pattern of glycerol decline occurring simultaneously with increasing ^{14}C levels at position I in the alanine and proline groups suggests that glycerol was converted into carbohydrate under these circumstances. This is consistent with the fact that glycogen levels increased under the same conditions. Little more can be said until further study can be done

to clarify the dynamics of these transformations.

Seasonal Trends in ^{14}C Distribution

Figure 9 displays data obtained from insects held outdoors at ambient temperatures for the winter. The 4 hemolymph samplings were done without allowing reacclimation to take place in order to determine the extent of ^{14}C redistribution in naturally acclimatized, overwintering insects. Unfortunately spring sampling was cut short because of an insufficient supply of ^{14}C fed insects at that time. This was due to normal winter mortality plus the fact that insufficient numbers were taken at the beginning of the experiments.

Comparing the bar graphs of proline, alanine, and glucose fed insects in Figure 9 one can make the generalization that ^{14}C administered as proline and glucose tended to remain in those substances with little seasonal shift from the original compounds. It appears here and in Figure 10 that ^{14}C administered as alanine more than proline ended up in glucose. In alanine there was a shift through January at least, of ^{14}C which appeared to move into the glycerol fraction. It seems that a reversal occurred sometime between January and March whereby glycerol carbon moved into alanine again. Proline, on the other hand, showed little tendency to be converted to glucose or glycerol, although the total amount of ^{14}C present in proline appeared to increase through January, followed by some decline by March 1. Curiously, ^{14}C levels in the glycerol fraction appeared to decrease slightly through January, then rose again by March 1, even though actual glycerol levels remained high throughout the entire period, such that the glycerol experienced

some degree of carbon turnover.

The glucose graph, although incomplete, indicates that glucose turnover may have been particularly high, that not a great amount of it was degraded to amino acids, but that ^{14}C from glucose did accumulate in the glycerol fraction.

Enzyme studies

Certain time and temperature related changes at the molecular level appear to occur generally in organisms which are undergoing seasonal acclimatization and although something is known about what occurs during transitional stages, and the new stage of acclimatization once completed can be characterized, little is understood of certain mechanisms of the process itself. In poikilotherms, the relative importance of changing photoperiod and temperature or their interaction as inducing stimuli in the changes that follow is not clearly known. Once the appropriate stimulus is perceived by the animal the response must be mediated by either neural or endocrine agents but the sequence or importance of each is not clear. However, we do know that during the transition to the new, altered state of acclimatization, altered rates of protein and nucleic acid synthesis occur, as well as changes in biosynthetic rates associated with activation and alteration of some metabolic pathways (Somero and Hochachka, 1971). Transitional phases of acclimatization must be periods during which isozymes are induced and when conformational changes take place in enzyme molecular structure to adapt them to changing thermal environments (Behrisch, 1969b, 1973; Iwatsuki and Okazaki, 1969).

In the final state of acclimatization we know that the organism is likely to possess different isozymes, ribosomes, and membrane lipids, altered ionic composition of body fluids and altered metabolic pools (Somero and Hochachka, ibid). Thus there is a change in both quantity and type of molecules present.

Such changes imply that enzyme systems are also involved in the restructuring and alteration of metabolic processes which accompany seasonal adaptation. Numerous studies have shown that this is the case. A review by Somero and Hochachka (1971) draws attention to some of the basic functions of enzymic systems required for adaptation to shifting temperatures. The primary requirement of a system is that the enzymes must function at temperatures to which the organism is adapted. In all systems studied, summer and winter variants of enzymes are found, which differ in their affinity for substrate, measured by the Michaelis constant or Km. By some biochemical mechanism it appears that enzymes are produced which exhibit the greatest affinity for their substrates (lowest Km) at the minimum temperatures experienced by the organisms in its habitat. This insures that over the temperature range to which the organism is adapted and at low substrate concentrations the enzymes can operate independently, without inactivation at temperature extremes. It appears that this enzyme-substrate affinity may be the most important factor in thermal acclimation at the enzyme level.

Enzymatic control of glycerol metabolism, and many other aspects of metabolism during cold acclimatization in insects is universally

conceded, thus it is surprising how little is actually known of the specific enzymes involved and the nature of the controlling mechanisms. Studies have shown the existence of appropriate enzymes to carry out the synthesis of glycerol and other polyols, but knowledge of controlling factors and of specific dynamics of this system remains extremely scanty when compared to some other well studied systems.

Faulkner (1956, 1958) found a dehydrogenase in silkworm blood which, in the presence of NADPH, had the capability to reduce some sugar phosphates, aldehydes and carboxyl compounds to their corresponding polyols. A single enzyme was reported to catalyze several reactions.

Chino (1960), utilizing dialyzed extract of silkworm eggs reported the presence of 4 separate polyol dehydrogenases, 2 of which were active in the production of glycerol, and 2 in the production of sorbitol. Chino (ibid) felt that during diapause of the silkworm egg a blockage of electron transport occurred, causing a buildup of NADPH. The abundance of hydrogen donors would favor the reduction instead of oxidation of sugars during these periods. In glycerol synthesis 2 enzymes were considered operative. One reduces glyceraldehyde or dihydroxyacetone in the presence of NADPH to glycerol and NADP, and the other reduces dihydroxyacetonephosphate (DHAP), with NADH to α-glycerophosphate and NAD under mediation of α-glycerolphosphate dehydrogenase (α-GDH). Since the work of Kubista (1958), Chefurka (1958), and Zebe and McShan (1967), it has been known that in most insects α-GDH more or less replaces lactic dehydrogenase in rapidly metabolising tissues such as flight muscles of bees and flies.

Chino (1960) also found a preponderance of α-GDH in silkworm eggs and considered this to be one of the factors contributing to glycerol accumulation. Interestingly, Chino (ibid) found that the polyol dehydrogenases were present in non-diapausing as well as diapausing eggs, even though polyols did not accumulate in the former. This was a major consideration in causing him to invoke a blockage of normal electron transport mechanisms during diapause.

Sømme and Velle (1968) studied polyol dehydrogenases in diapausing pupae of a cabbage butterfly (Pieris) and found no evidence for more than one enzyme in this species. That conclusion was based on the fact that the activity curves with different substrates are very similar. This does not seem surprising since only sorbitol accumulates in Pieris.

The question of the exact number of enzymes present which accomplish the various reactions is still open. However, I would consider this to be relatively unimportant until we understand more than we do at the present time about controlling mechanisms in glycerol accumulation.

After Chino (1960) demonstrated the presence of necessary dehydrogenases in silkworm eggs to reduce sugar to either sorbitol-6-phosphate or α-glycerolphosphate, he then showed that the eggs possessed the enzymatic capability for splitting these phosphate esters to their corresponding polyols. He postulated that a single enzyme with an optimum pH of 6.7 was responsible for the hydrolysis of both compounds.

Wyatt (1967) mentions that Wyatt and Thomas (unpub.) found preliminary evidence for a specific α-glycerophosphatase in fat body tissue

of Cecropia pupae. Supporting evidence for this possibility includes the fact that Lenartowicz (1961) found that during cold exposure the level of free inorganic phosphate increased in larvae of Galleria, the wax moth, while levels of α-glycerophosphate decreased. It is somewhat puzzling therefore to discern the reason for my inability to find α-glycerophosphatase activity in P. brevicornis. My methods were substantially the same as those of Chino (1961) and a wide range of pH and substrate concentration were tried on many different extracts from winter acclimatized, adult beetles. It is still possible that the right conditions were not used or that the enzyme somehow became deactivated in the process. It may also be that the process is not enzymatically regulated at this step. Due to the brevity of this particular phase of the research it is not possible to state the reason with certainty.

Nordin et al. (1970), in a study of glycerol accumulation in hibernating carpenter ants (Camponotus), examined a number of glycolytic enzymes including glyceraldehyde-3-phosphate dehydrogenase, triose phosphate isomerase, α-glycerophosphatase, and α-glycerophosphate dehydrogenase. They could find no difference in the relative activities between the enzymes of summer and hibernating ants, although in the paper no data are presented except for glyceraldehyde-3-phosphate dehydrogenase.

Fructose Disphosphatase

FDPase was studied because of its key position at a branch point in glycolytic metabolism, because it has been well studied by others

(Behrisch, 1969a and b, 1971, 1972; Behrisch and Hochachka, 1969a and b), and because the facilities at the laboratory of Dr. H. Behrisch were set up for the study of phosphatase enzymes.

FDPase has been characterized (Krebs, 1963; Horecker et al., 1966; Newsholme and Gevers, 1967), as an enzyme which catalyzes a rate limiting step in gluconeogenesis and whose regulation carries important implications for carbohydrate metabolism (Behrisch, 1973). Briefly, and with reference to Figure 16, if FDPase is active, the result is a net carbon flow into glycogen, or a metabolism which results in net energy storage, whereas if FDPase is inactive and phosphofructokinase, which mediates the hydrolysis of inorganic phosphate from FDP to yield F-6-P is active instead, carbon flows from storage carbohydrate into energy yielding pathways. Therefore a major regulatory role could be played by FDPase over carbohydrate metabolism at this step and it is for this reason that much effort has gone into understanding the properties and functions of the enzyme.

In the present study, using dialyzed extracts of winter acclimatized P. brevicornis, we briefly looked at the relative activity of the enzyme over a range of cofactor concentrations with respect to temperature of 0°C and 15°C, and pH values of 7.5, 8.5 and 9.5 (Fig. 12). It is interesting that at 0°C the enzyme was apparently more active at pH 7.5 than at the pH 8.5, whereas at 15°C, far greater amounts of activity were attained at pH 8.5 than pH 7.5. However, it must be noted that under lower cofactor concentrations, from 1×10^{-6} to 1×10^{-5} M Mn^{+2}, pH 8.5 showed more activity at both low and high temperature. If intracellular

Mn^{+2} concentrations are actually in this lower range, then pH 8.5 would be the optimum for both 0 and 15°C.

Alkaline pH optima may be a general feature of at least arctic poikilotherm enzymes. This could be an adaptive feature since lower environmental temperatures cause an increase in intracellular pH because of the dependence on temperature of the dissociation constant of water (Behrisch, 1973). Thus the enzymes could be fully functional when decreasing temperatures cause pH increases within the cell.

It would be of great interest to conduct further enzyme studies at even lower temperatures, possibly in supercooled solutions if the technical difficulties in doing so could be solved.

Unfortunately, it was not possible to accomplish more with enzymes involved in glycerol synthesis or in the cold hardening process.

SUMMARY AND CONCLUSIONS

1. Glycerol and Glycogen Levels. Winter concentrations of glycerol in P. brevicornis reached maximum values of 12-13% of hemolymph volume. Concentrations began to increase in the fall after freezing began to occur at night, attained maximum levels in the 8-13% range and remained fairly constant until spring.

Cyclic variations in whole body glycogen content suggest that an interconversion can take place between glycerol and glycogen. Stoichiometric analysis of this conversion was not attempted although ^{14}C isotope study indicated that interconversion of carbon molecules took place between glycerol and glycogen.

2. Amino Acids. Analysis of free hemolymph amino acids showed that heavy concentrations (.425 M yearly average) were present year around, and that proline was by far the most abundant of those found. Significant seasonal variation was not demonstrated in either total concentration of amino acids or in the concentration of any of the more common acids.

3. Fats and Fatty Acids. Fat content was measured seasonally as a percent of dry body weight in pooled insect samples. An apparent trend toward decreasing fat content during hibernation was noted, although the decline is not marked (less than 10%).

Fatty acids were measured seasonally and at 2 different levels of temperature adaptation. A marked desaturation is evident during winter hibernation and with cold versus warm temperature acclimation.

4. Radioisotope Feeding Experiments. Carbon transfer during hibernation was suggested by changing ^{14}C levels in at least three metabolite fractions. It also appeared that carbon incorporated into glycerol can originate from carbohydrate and amino acid pools at least, and that interconversion of carbon took place between the three compartments.

5. Enzyme activity was briefly studied to determine the relative activities of fructose diphosphatase and α-glycerophosphatase. No α-glycerophosphatase activity could be demonstrated. FDPase showed activity similar to that reported in other poikilotherm enzyme systems, at least in the Mn^{+2} saturation curves. There was an apparent alkaline pH optimum of 8.5.

6. Microclimate measurements of P. brevicornis suggested that temperatures in the preferred winter microhabitat were effectively moderated by snow cover, moss, and punky wood layers. Temperatures below -20°C were not recorded even during the coldest winter periods.

7. Conclusions. The seasonal cycle of glycerol accumulation is accompanied by other metabolic changes, some of which may contribute significantly to winter cold hardening. Glycerol in itself is not sufficient as a cryoprotectant to insure winter survival, and most likely acts in concert with other processes such as lipid saturation changes to insure this end. Glycerol concentrations were found not to reach the high levels previously reported for this species. Aerobic metabolism appears to continue even while the insects are experiencing extreme cold. Metabolic conversions and carbon transfer apparently

take place throughout hibernation also.

It is suggested that cold hardening and winter survival in P. brevicornis involves restructuring of many metabolic processes, but as yet the details and mechanisms of these changes are not understood.

REFERENCES

Ackman, R. G. 1963. Structural correlation of unsaturated fatty acid esters through graphical comparison of gas-liquid chromatographic retention times on a polyester substrate. J. Am. Oil Chem. Soc. 44:558-564.

Adkisson, P. L. 1966. Internal clocks and insect diapause. Science 154:234-241.

Asahina, E. 1966. Freezing and frost resistance in insects. In Cryobiology (H. Meryman, ed.). Academic Press, New York.

Asahina, E. 1966. Unpublished observations.

Atkinson, D. E. 1966. Regulation of enzyme activity. Ann. Rev. Biochem. 35:85-124.

Bade, M. L. 1962. Metabolic conversions during pupation of the cecropia silkworm. 2. Tests for the operation of the glyoxylate cycle. Biochem. J. 83:478-482.

Bade, M. L. and G. R. Wyatt. 1962. Metabolic conversions during pupation of the cecropia silkworm. I. Deposition and utilization of nutrient reserves. Biochem. J. 83:470-478.

Ball, G. E. 1963. The distribution of the species of subgenus Cryobius (Coleoptera, Carabidae, Pterostichus) with special reference to the Bering Land Bridge and Pleistocene refugia. In Pacific Basin Biogeography (J. L. Gressit, ed). 10th Pac, Sci. Conf., Bishop Museum Press.

Barker, R. J. and Y. Lehner. 1972. Free amino acids in thoraces of flown honeybees, Apis mellifera L. (Hymenoptera:Apidae). Comp. Biochem. Physiol. 43B:163-169.

Barlow, J. S. 1964. Fatty acids in some insect and spider fats. Can. J. Biochem. 42:1365-74.

Bataillon, E. and E. Couvreur. 1892. La function glycogenique chez le ver à soie pendant la métamorphose. C. r. Séanc. Soc. Biol. Sér 9, 4:669-671.

Baust, J. and L. Nauman. 1969. Unpublished observations.

Baust, J. G. 1970. Influences of low temperature on cold hardiness and neural adaptation in the Alaskan beetle, Pterostichus brevicornis (Carabidae). Ph.D. Thesis, Univ. of Alaska.

Baust, J. G. and L. K. Miller. 1970. Variations in glycerol content and its influence on cold hardiness in the Alaskan carabid beetle, Pterostichus brevicornis. J. Insect Physiol. 16:979-990.

Baust, J. G. and L. K. Miller. 1972. Influence of low temperature acclimation on cold hardiness in the beetle, Pterostichus brevicornis. J. Insect Physiol. 18:1935-1948.

Beck, S. D. 1968. Insect Photoperiodism. Academic Press, New York.

Behrisch, H. W. 1969a. Temperature and the regulation of enzyme activity in poikilotherms. Regulatory properties of fish fructose diphosphatase. Ph.D. Thesis, Univ. of British Columbia.

Behrisch, H. W. 1969b. Temperature and the regulation of enzyme activity in poikilotherms. Fructose diphosphatase from migrating salmon. Biochem. J. 115:687-696.

Behrisch, H. W. 1973. Molecular mechanisms of temperature adaptation in arctic ectotherms. In Effects of Temperature on Ectothermic Organisms (W. Wieser, ed). Springer Verlag, New York. pp. 123-137.

Behrisch, H. W. and P. W. Hochachka. 1969a. Temperature and the regulation of enzyme activity in poikilotherms. Properties of rainbow trout fructose diphosphatase. Biochem. J. 111:287-295.

Behrisch, H. W. and P. W. Hochachka. 1969b. Temperature and the regulation of enzyme activity in poikilotherms. Properties of lungfish fructose diphosphatase. Biochem. J. 112:601-607.

Buffington, J. D. and J. H. Zar. 1968. Changes in fatty acid composition of Culex pipiens pipiens during hibernation. Ann. Entom. Soc. Am. 61:774-775.

Burkett, B. and H. Schneidermann. 1968. Coordinated neuromuscular activity in insect spiracles at subzero temperatures. Nature 217: 95-96.

Bursell, E. 1963. Aspects of the metabolism of amino acids in the tsetse fly, Glossina (Diptera). J. Insect Physiol. 9:439-452.

Carpenter, W. D. and E. G. Jaworski. 1962. The presence of isocitrate lyase activity in the southern army worm, Prodenia eridania. Biochem. biophys. Acta 58:369-371.

Chefurka, W. 1958. Glucose metabolism in insects. Symposium XII, Biochemistry of Insects, pp. 115-137. The IVth Int. Congress of Biochemistry, Vienna. Pergamon Press, London.

Chino, H. 1957. Conversion of glycogen to sorbitol and glycerol in the diapause egg of the Bombyx silkworm. Nature 180:606-607.

Chino, H. 1958. Carbohydrate metabolism in the diapause egg of the silkworm, Bombyx mori. II. Conversion of glycogen into sorbitol and glycerol during diapause. J. Insect Physiol. 2:1-12.

Chino, H. 1960. Enzymatic pathways in the formation of sorbitol and glycerol in the diapausing egg of the silkworm, Bombyx mori. I. On the polyol dehydrogenases. J. Insect Physiol. 5:1-15.

Chino, H. 1961. Enzymatic pathways in the formation of sorbitol and glycerol in the diapausing egg of the silkworm, Bombyx mori. II. On the phosphatases. J. Insect Physiol. 6:231-240.

Crowe, J. H. and A. F. Cooper, Jr. 1971. Cryptobiosis. Sci. Amer. 225:30-36.

Deuel, H. J. and M. G. Morehouse. 1946. The interrelations of carbohydrate and fat metabolism. In Advances in Carbohydrate Chemistry (W. W. Pigman and M. L. Wolfrom, eds.). Vol. 2, pp. 119-160. Academic Press, New York.

Downes, J. A. 1965. Adaptations of insects in the Arctic. Ann. Rev. Entom. 10:257-274.

Dubach, P., F. Smith, D. Pratt, and C. M. Stewart. 1959. Possible role of glycerol in the winter hardiness of insects. Nature 184:288-289.

Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for the determination of sugars and related substances. Anal. Chem. 28:350-356.

Fast, P. G. 1964. Insect lipids: a review. Mem. Entom. Soc. Can. 37:5-50.

Faulkner, P. 1956. Enzymic reduction of sugar phosphates in insect blood. Biochem. J. 64:436-441.

Faulkner, P. 1958. Polyol dehydrogenase in the silkworm. Biochem. J. 68:374-380.

Fiske, C. H. and Y. Subbarow. 1925. Colorimetric determination of phosphorus. J. Biol. Chem. 66:375.

Florkin, M. and Ch. Jeuniaux. 1964. Hemoymph: Composition. In The Physiology of Insects (M. Rockstein, ed.). Vol 3, Chap. 2. Academic Press, New York.

Fox, C. F. 1969. A lipid requirement for induction of lactose transport in Escherichia coli. Proc. Nat. Acad. Sci. U.S. 63:850.

Fox, C. F. 1972. The structure of cell membranes. Sci. Amer. 226: 30-38.

Gilbert, L. I. 1967. Lipid metabolism and function in insects. In Advances in Insect Physiology (J. W. L. Beament, J. E. Treherne, and V. B. Wigglesworth, eds.) Vol. 4, pp. 69-211. Academic Press, New York.

Harvey, W. R. 1962. Metabolic aspects of insect diapause. Ann. Rev. Entom. 7:57-80.

Harwood, R. F. and N. Takata. 1965. Effect of photoperiod and temperature on fatty acid composition of the mosquito, Culex tarsalis. J. Insect Physiol. 11:711-716.

Hazel, J. R. 1973. The regulation of cellular function by temperature-induced alterations in membrane composition. In Effects of Temperature on Ectothermic Organisms (W. Wieser, ed.). pp. 54-67. Springer Verlag, New York.

Hochachka, P. W. 1967. Organization of metabolism during temperature compensation. In Molecular Mechanisms of Temperature Adaptations (C. L. Prosser, ed). AAAS Publ. 84, Washington, D.C. pp. 177-203.

Horecker, B. L. and S. Pontremoli, S. Rosen, and O. Rosen. 1966. Structure and function in fructose diphosphatase. Fed. Proc. 25: 1521-1528.

House, H. L., D. F. Riodan, and J. S. Barlow. 1958. Effects of thermal conditioning and of degree of saturation of dietary lipids on resistance of an insect to a high temperature. Can. J. Zool. 36: 629-632.

Iwatsuki, N. and R. Okazaki. 1967. Mechanisms of regulation of deoxythymidine kinase in Escherichia coli. II. Effect of temperature on the enzyme's activity and its kinetics. J. Mol. Biol. 29:155-165.

Jacob, F. and J. Monod. 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. 3:318-356.

Jacob, F. and J. Monod. 1963. Genetic repression, allosteric inhibition, and cellular differentiation. Symp. Soc. Study Develop. Growth 21(1962):30-64.

Janetschek, H. 1948. Tierische Succession auf hochalpinen Neuland. Schiernschriften 67:1-215.

Karnavar, G. K. and K. S. S. Nair. 1969. Changes in body weight, fat, glycogen, and protein during diapause of Trogoderma granarium. J. Insect Physiol. 15:95-103.

Kaufmann, T. 1969. Life history of Upis ceramboides at Fairbanks, Alaska. Ann. Entom. Soc. Amer. 69:922-923.

Kaufmann, T. 1969. Ecobiological aspects of hibernation in the arctic beetle, Pterostichus brevicornis (Coleoptera:Carabidae) in Alaska. (unpublished)

Kaufmann, T. 1971. Hibernation in the arctic beetle, Pterostichus brevicornis, in Alaska. J. Kansas Entom. Soc. 44:81-92.

Kennedy, E. P. 1961. Biosynthesis of complex lipids. Fed. Proc. 20:934-940.

Kilby, B. A. 1963. The biochemistry of the insect fat body. In Advances in Insect Physiology (J. W. L. Beament, J. E. Treherne and V. B. Wigglesworth, eds.). Vol. 1:111-174. Academic Press, New York.

Krebs, H. A. 1963. Renal gluconeogensis. Advan. Enzyme Regulation 1:385-400.

Krunic, M. D. and R. W. Salt. 1971. Seasonal changes in glycerol content and supercooling points of Megachile rotundata (F.) and M. relativa Cress. Can. J. Zool. 49:663-666.

Kubista, V. 1958. Anaerobe Glykolyse in den Insektenmuskeln. Biochem. Z. 330:315-323.

Kubista, V. and Z. Bartos. 1960. Free and fixed glycogen in insect muscle. Physiologia Bohemoslov. 9:235-239.

Kurland, C. G., H. A. Schneiderman, and R. D. Smith. 1958. Oxygen debts in diapausing insects. Anat. Rec. 132:465-466.

Lambremont, E. N., M. S. Blum, R. M. Schrader. 1964. Storage and fatty acid composition of triglycerides during adult diapause of the boll weevil. Ann. Entom. Soc. Amer. 57:526-532.

Lenartowicz, E. 1961. Effect of low temperature upon phosphorus metabolism in Galleria mellonella larvae. Acta Biochem. pol. 8:15-24.

Levitt, J. 1966. Frost hardening in higher plants accompanied by increase in cell permeability to water. In Cryobilogy (H. T. Meryman, ed.). Academic Press, New York. pp. 235-246.

Lue, P. F. and S. E. Dixon. 1967. Studies in the mode of action of royal jelly in honeybee development. VII. The free amino acids in the hemolymph of developing larvae. Can. J. Zool. 45:205-214.

Mani, M. S. 1962. Introduction to high altitude entomology. Methuen and Co., Ltd., London. 302 p.

Mansingh, A. and B. N. Smallman. 1971. The influence of temperature on the photoperiodic regulation of diapause in Saturniids. J. Insect Physiol. 17:1735-1739.

Mazur, P. 1969. Freezing injury in plants. Ann. Rev. Plant Physiol. 20:419-448.

Mazur, P. 1970. Cryobiology: the freezing of biological systems. Science 168(3934):939-949.

Metcalfe, L. D. and A. A. Schmitz. 1961. The rapid preparation of fatty acid esters for gas chromatographic analysis. Anal. Chem. 33:363-364.

Miller, L. K. 1969. Freezing tolerance in an adult insect. Science 166:105-106.

Murphy, J. B. and M. W. Kies. 1960. Spectrophotometric determination of proteins in dilute solutions. Biochem. Biophys. Acta 45:382.

Newsholme, E. A. and W. Gevers. 1967. Control of glycolysis and gluconeogenesis in liver and kidney cortex. Vitamines and Hormones 25: 1-87.

Nordin, J. H., R. Duffield, N. Friedman, W. Gelb and J. F. Brandt. 1970. Enzyme activity in cryobiological systems. Cryobiol. 6:373-383.

Overath, P., H. U. Schairer, and W. Stoffel. 1970. Correlation of in vivo and in vitro phase transitions of membrane lipids in Escherichia coli. Proc. Nat. Acad. Sci. 67(2):606-612.

Pantyukov, G. A. 1964. The effect of low temeprature on different populations of the brown tail moth Euproctis chrysorrhoea (L.) and the gypsy moth Lymantria dispar (L.) (Lepidoptera, Orgyidae). Ent. Rev. Wash. 43:47-55.

Passey, R. F. and D. Fairbairn. 1957. The conversion of fat to carbohydrate during embryonation of Ascaris eggs. Can. J. Biochem. Physiol. 35:511-525.

Perkins, H. J. and S. Aronoff. 1959. A paper chromatographic method for the purification of shikimic acid-u-C^{14} obtained from culture filtrates of a mutant of Escherichia coli. Can. J. Biochem. Physiol. 37:149-150.

Piez, K. A. and L. Morris. 1960. A modified procedure for the automatic analysis of amino acids. Anal. Biochem. 1:187-201.

Polge, C., A. U. Smith, and A. S. Parkes. 1949. Revival of spermatozoa after vitrification and dehydration at low temperature. Nature 164:666.

Sacktor, G. 1965. Metabolism of amino acids in muscle. In The Physiology of Insects (M. Rockstein, ed.). Vol. 2, pp. 525-528. Academic Press, New York.

Sacktor, B. and C. C. Childress. 1967. Metabolism of proline in insect flight muscle and its significance in stimulating the oxidation of pyruvate. Arch. Biochem. Biophys. 120:583-588.

Salt, R. W. 1957. Natural occurrence of glycerol in insects and its relation to their ability to survive freezing. Can. Entomol. 84: 491-494.

Salt, R. W. 1958. Relationships of respiration rate to temperature in a supercooled insect. Can. J. Zool. 36:265-268.

Salt, R. W. 1959. Role of glycerol in the cold hardening of Bracon cephi (Graham). Can. J. Zool. 37:59-69.

Salt, R. W. 1961. Principles of insect cold hardiness. Ann. Rev. Entomol. 6:55-74.

Saunders, D. S. 1970. Circadian clock in insect photoperiodism. Science 168:601-603.

Saunders, D. S. 1971. The temperature-compensated photoperiodic clock "programming" development and pupal diapause in the flesh fly, Sarcophaga argyrostoma. J. Insect. Physiol. 17:801-812.

Saz, H. J. 1969. Carbohydrate and energy metabolism of nematodes and acanthocephala. In Chemical Zoology (M. Florkin and B. T. Scheer, eds.). Vol. III, pp. 329-360. Academic Press, New York.

Saz, H. J. 1971. Facultative anaerobiosis in the invertebrates: pathways and control systems. Amer. Zoologist 11:125-135.

Saz, H. and E. Bueding. 1966. Relationships between anthelmintic effects and biochemical and physiological mechanisms. Parmacol. Rev. 18:871-894.

Saz, H. J. and J. A. Hubbard. 1957. The oxidative decarboxylation of malte by Ascaris lumbricoides. J. Biol. Chem. 225:921-933.

Saz, H. J. and O. L. Lescure. 1967. Glyconeogeneis, fructose 1,6-diphosphatase and phosphoenolpyruvate carboxykinase activities of Ascaris lumbricoides adult muscle and larvae. Comp. Biochem. Physiol. 22:15-28.

Saz, H. J. and O. L. Lescure. 1969. The functions of phophoenol-pyruvate carboxykinase and malic enzyme in the anaerobic formation of succinate by Ascaris lumbricoides. Comp. Biochem. Physiol. 30:49-60.

Saz, H. J. and A. Vidrine, Jr. 1959. The mechanism of formation of succinate and propionate by Ascaris lumbricoides muscle. J. Biol. Chem. 234:2001-2005.

Scholander, P. F., W. Flagg, R. J. Hock, and L. Irving. 1953. Studies on the physiology of frozen plants and animals in the Arctic. J. Cell Comp. Physiol. 42, Suppl. 1, 1-56.

Somero, G. N. and P. W. Hochachka. 1971. Biochemical adaptation to the environment. Amer. Zool. 11:157-165.

Sømme, L. 1964. Effects of glycerol on cold hardiness in insects. Can. J. Zool. 2:87-101.

Sømme, L. 1966. The effect of temperature, anoxia, or injection of various substances on hemolymph composition and supercooling in larvae of Anagosta kuehniella (Zell.). J. Insect Physiol. 12: 1069-1083.

Sømme, L. 1967. The effect of temperature and anoxia on haemolymph composition and supercooling in 3 overwintering insects. J. Insect Physiol. 13:805-814.

Sømme, L. and W. Velle. 1968. Polyol dehydrogenase in diapausing pupae of Pieris brassicae. J. Insect Physiol. 14:135-143.

Spackman, D. H., W. H. Stein, and S. Moore. Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem. 30:1190-1206.

Steim, J. M., M. E. Tourtellote, J. C. Reinert, R. N. McElhaney, and R. L. Rader. 1969. Calorimetric evidence for the liquid-crystalline state of lipids in a biomembrane. Proc. Natl. Acad. Sci. 63:104-109.

Stevenson, E. and G. R. Wyatt. 1964. Glycogen phosphorylase and its activation in silkmoth fat body. Arch. Biochem. Biophys. 108: 420-429.

Takehara, I. 1966. Natural occurrence of glycerol in the slug caterpillar Monema flavescens. Contrib. Inst. Low Temp. Sci. Ser. B 14:1-34.

Takehara, I. and E. Asahina. 1960. Frost-resistance and glycerol content in overwintering insects. (Japanese, English Summary). Low Temp. Sci. Ser. B. 18:57-65.

Tanno, K. 1962. Frost resistance in a carpenter ant, Camponotus obscuripes obscuripes. I. The relation of glycerol to frost resistance. Low Temp. Sci. Ser. B 20:25-34.

Tombes, A. S. 1966. Aestivation (summer diapause) in Hypera postica (Coleoptera: Curculionidae). I. Effect of aestivation, photoperiods, and diet on total fatty acids. Ann. Entomol. Soc. Amer. 59:376-380.

Twente, J. W. and J. Twente. 1970. Arousing effects of trophic hormones in hibernating Citellus lateralis. Comp. Gen. Pharmacol. 1:431-436.

Weis-Fogh, T. 1967. Metabolism and weight economy in migrating animals, particularly birds and insects. In Insects and Physiology (J. W. L. Beament and J. E. Treherne, eds.). pp. 143-159. Academic Press, New York.

Wilhelm, R. C., H. A. Schneiderman, and L. J. Daniel. 1961. The effect of anaerobiosis on the giant silkworm Hyalophora cecropia and Samia cynthia with special reference to the accumulation of glycerol and lactic acid. J. Insect Physiol. 7:273-288.

Wilson, G., S. P. Rose and C. F. Fox. 1970. The effect of membrane lipid unsaturation on glycoside transport. Biochem. Biophys. Res. Commun. 38:617-623.

Wyatt, G. R. 1963. Metabolic regulation in the development of insects. In Control Mechanisms in Respiration and Fermentation (B. Wright, ed.). pp. 179-188. Ronald Press, New York.

Wyatt, G. R. 1967. The chemistry of sugar and polysaccharides in insects. In Advances in Insect Physiology (J. W. L. Beament, J. E. Treherne, and V. B. Wigglesworth, eds.). Vol. 4, pp. 287-347. Academic Press, New York.

Wyatt, G. R. and G. F. Kalf. 1957. The chemistry of insect hemolymph. II. Trehalose and other carbohydrates. J. Gen. Physiol. 40:833-847.

Wyatt, G. R. and G. F. Kalf. 1958. Organic compounds of insect hemolymph. In Fed. Proc. 10th Int. Cong. on Entomology, Montreal. Vol. 2, p. 333.

Wyatt, G. R. and W. L. Meyer. 1959. The chemistry of insect hemolymph. III. Glycerol. J. Gen. Physiol. 42:1005-1011.

Wyatt, G. R. and B. P. Thomas. 1967. Unpublished.

Zaluska, H. 1959. Glycogen and chitin metabolism during development of the silkworm (Bombyx mori L.). Acta biol. exp., Lodz 19:339-351.

Zebe, E. C. and W. H. McShan. 1957. Lactic and alpha-glycerophosphate dehydrogenase in insects. J. Gen. Physiol. 40:779-783.

Zweig, G. and J. Sherma (eds.). 1972. Handbook of Chromatography. Vol. I. CRC Press, Cleveland, Ohio.